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(54) Expression of human serum albumin in *pichia pastoris*.

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(57) A novel expression cassette, vectors and process for the secretion of HSA in *Pichia pastoris* cells. In accordance with the present invention there has been discovered an improved expression cassette for the production of HSA in *Pichia pastoris* comprising  
 a) a *Pichia pastoris* 5' regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 5' regulatory region and the *Pichia pastoris* DAS1 5' regulatory region wherein the 3' end of the 5' regulatory region is operably linked to;  
 b) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 11 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to  
 c) a 3' termination sequence.

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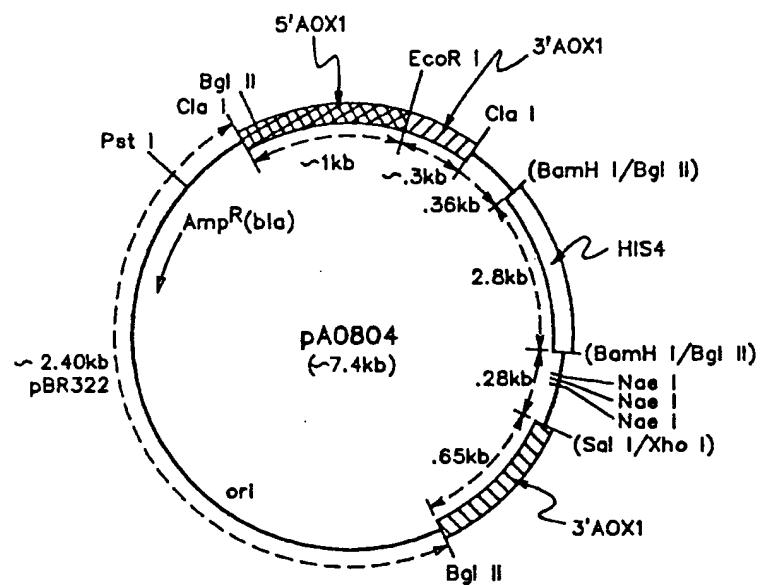


FIG. 1

Field of the Invention

This invention relates to the field of recombinant DNA biotechnology. In one aspect, this invention relates to a process for the expression of human serum albumin (HSA) in *Pichia pastoris*.

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Background

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Human serum albumin is the most abundant plasma protein of adults. The concentration of albumin is 40 mg/ml, or 160g of albumin circulating throughout the human body for a 70 kg adult male. This protein maintains osmotic pressure and functions in the binding and transport of copper, nickel, calcium (weakly, at 2-3 binding sites), bilirubin and protoporphyrin, long-chain fatty acids, prostaglandins, steroid hormones (weak binding with these hormones promotes their transfer across the membranes), thyroxine, triiodothyronine, and glutathione. According to Peters, T. and Reed, R.G. in *Albumin: Structure, Biosynthesis and Function*, (Peters, T. and Sjoholm, J. eds.) 1977 p.11-20, over 10,000 kilograms of purified albumin are administered annually in the United States alone to patients with circulatory failure or with albumin depletion.

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Currently the only commercial source of HSA is from fractionated blood. Considering the possible dangers of blood borne contaminants and pathogens, it would be a considerable contribution to the commercial production of HSA to develop alternate methods of producing HSA. With the advent of recombinant DNA technology, it is now possible to produce HSA by alternate methods.

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HSA has also been expressed in *Saccharomyces cerevisiae* as disclosed by Etcheverry et al. in *Bio/technology*, August 1986, p. 726 and Arjum Singh in EPA 123,544. Etcheverry disclosed HSA expression intracellularly in a concentration of approximately 6 mg/l and the presence of cell-associated HSA. Hayasuke et al. also disclosed the expression of HSA in *Saccharomyces cerevisiae* in combination with the GAL 1 promoter and a signal sequence. Hayasuke et al. appears to have been able to achieve a secreted production level of 160 mg/l. As described in EPA344,459, HSA has also been expressed in *Pichia pastoris* as intracellular or cell-associated protein. Although the expression of HSA in yeast cells, such as *Saccharomyces cerevisiae* and *Pichia pastoris*, is a significant step toward providing safe alternative sources of HSA, the expression of HSA as an intracellular or cell-associated protein is not desirable. Expensive and time consuming measures are required to recover and purify intracellular or cell-associated HSA. Furthermore, it would also be advantageous if a high level of HSA secretion could be achieved to improve the yields and lower the production costs.

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Thus, it would be a significant contribution to the art to provide yeast strains which secrete high levels of HSA.

It also would be a significant contribution to the art to develop a process which produces HSA in a manner that is easy to recover and purify.

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Therefore, it is an object of this invention to provide strains which produce high levels of HSA. It is a further object of this invention to provide a process which produces HSA in a manner that is easy to recover and purify.

Other objects and advantages of the present invention will be apparent to those skilled in the art from the present specification.

Summary of the Invention

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In accordance with the present invention there has been discovered an improved expression cassette for the production of HSA in *Pichia pastoris* comprising

- a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to
- an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to
- a 3' termination sequence.

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In accordance with the present invention there has also been discovered *Pichia pastoris* strains transformed with an improved expression cassette for the production of HSA in *Pichia pastoris* comprising

- a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to

b) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to  
 c) a 3' termination sequence.

5 In a further embodiment of the present invention, there has also been discovered a process for the secretion of HSA from transformed *Pichia pastoris* cells comprising

a) transforming *Pichia pastoris* with at least one vector having at least one expression cassette comprising

i) a 5' *Pichia pastoris* regulatory region having a 5' end and a 3' end selected from the group consisting of the *Pichia pastoris* AOX1 regulatory region and the *Pichia pastoris* DAS1 regulatory region wherein the 3' end of the regulatory region is operably linked to

10 ii) an HSA structural gene encoding a signal sequence and mature protein having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within about 6 deoxyribonucleotides of the 5' end of said HSA structural gene; and operably linked to

15 iii) a 3' termination sequence; and then

b) culturing the resulting transformed *Pichia pastoris* under suitable conditions to obtain the secretion of HSA.

#### Detailed Description of the Figures

20 Figure 1 provides a representation of plasmid pA0804 which contains a linear site-specific integrative vector in the fragment clockwise from BglII to BglII. The structural gene may be inserted in the unique EcoRI site of this plasmid. This plasmid may be recovered from the plasmid DNA of NRRL B-18114 by EcoRI digest and gel electrophoresis to recover a linear ~7.4 kb EcoRI fragment corresponding to Figure 1.

25 Figure 2 provides a representation of pHSA13 in circular form.

Figure 3 provides a restriction map of the AOX1 5' regulatory region isolated from *Pichia pastoris*.

Figure 4 provides a restriction map of the DAS1 5' regulatory region isolated from *Pichia pastoris*.

Figure 5 provides a restriction map of the AOX1 3' termination sequence isolated from *Pichia pastoris*.

Figure 6 provides a restriction map of the DAS1 3' termination sequence isolated from *Pichia pastoris*.

30 Figure 7 provides a representation of pHSA113 in linear form.

Figure 8 provides a representation of plasmid pA0807N which contains a linear site-specific integrative vector in the fragment clockwise from NotI to NotI. The structural gene may be inserted in the unique EcoRI site of this plasmid.

#### Detailed Description

The present invention provides improved expression cassettes for the expression of HSA, improved vectors and *Pichia pastoris* strains transformed with these improved cassettes and vectors.

Utilizing the present invention, HSA secretion levels of approximately 1-3.4 grams of authentic HSA per 40 liter of fermentation broth have been obtained with an additional 15-88 µg HSA per mg of protein being present within the cell secretory pathway. This invention thus provides a means for the high level secretion of HSA. Achieving these levels of HSA production is a significant advancement over the prior production levels, since at the level of 1-3.4 grams per liter the recovery of HSA in high yields with high purities is possible.

45 To express the HSA structural gene, the gene must be operably linked to a 5' regulatory region and a 3' termination sequence, which forms an expression cassette which will be inserted into a host (usually a microorganism) via a vector (such as a circular plasmid or linear site-specific integrative vector). Operably linked as used in this context refers to a juxtaposition wherein the 5' regulatory region, structural gene, and 3' termination sequence are linked and configured so as to perform their normal function. 5' regulatory 50 region or promoter as used herein means DNA sequences which respond to various stimuli and provide enhanced rates of mRNA transcription. 3' termination sequences are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked (such as sequences which elicit polyadenylation). For the practice of this invention, it is preferred that the ATG of the structural gene be linked with as few intervening deoxyribonucleotides as possible to the 3' end of the 5' regulatory region, preferably about 11 or less deoxyribonucleotides and most preferably 8 or less deoxyribonucleotides. It is also preferred that the adenine and thymine content of the intervening deoxyribonucleotides be in the range of from about 55 percent to about 64 percent. Further, it appears that there are nucleotide preferences for certain specific

locations. Counting left from the ATG codon of the structural gene with the first position left being the -1 position it appears that adenine or cytosine is the most preferred deoxyribonucleotide, in the -2 position the most preferred deoxyribonucleotide is either adenine or thymine, in the -3 position the most preferred deoxyribonucleotide is adenine or thymine and the most preferred nucleotide at the -4 position is adenine, thymine or cytosine. Currently, it is preferred that the AOX1 or DAS1 5' regulatory regions having the restriction maps of Figures 3 and 4 or, the sequences provided as SEQ ID No: 1 and SEQ ID No: 2, respectively, be linked at their 3' end of the sequence to the ATG start codon of the HSA structural gene. Two examples of appropriate linkages for the AOX1 5' regulatory region are illustrated below.

Construct Designation	End of the 5' Regulatory Region for <u>AOX1</u>	Deoxyribonucleotide intervening before ATG start codon
pHSA140	5' - TTCGAAACG	5' - AGGAATTC
pHSA413, pHSA313	5' - TTCGAAACG	5' - NONE

Several regulatory regions have been characterized and can be employed in conjunction with the expression of HSA in *Pichia pastoris*. Exemplary 5' regulatory regions are the primary alcohol oxidase (AOX1), dihydroxyacetone synthase (DAS1), and the p40 regulatory regions, derived from *Pichia pastoris* and the like. The presently preferred 5' regulatory regions employed in the practice of this invention are those characterized by their ability to respond to methanol-containing media, such regulatory regions selected from the group consisting of AOX1, and DAS1, disclosed by D. W. Stroman et al. in U.S. Patent 4,855,231, incorporated herein by reference. The most preferred 5' regulatory region for the practice of this invention is the AOX1 5' regulatory region.

3' termination sequences should be utilized in the expression cassette as discussed above. 3' termination sequences may function to terminate, polyadenylate and/or stabilize the messenger RNA coded for by the structural gene when operably linked to a gene, but the particular 3' termination sequence is not believed to be critical to the practice of the present invention. A few examples of illustrative sources for 3' termination sequences for the practice of this invention include but are not limited to the *Hansenula polymorpha* and *Pichia pastoris* 3' termination sequences. Preferred are those derived from *Pichia pastoris* such as those selected from the group consisting of the 3' termination sequences of AOX1 gene, DAS1 gene, p40 gene and HIS4 gene. Particularly preferred is the 3' termination sequence of the AOX1 gene.

*Pichia pastoris* may be transformed with a variety of HSA structural genes (in the inventive transformants discussed herein the HSA structural gene encodes both a signal sequence and a mature HSA protein). HSA structural genes have been sequenced by Lawn et al. Nuc. Acids Res. 9:6105 (1981), and Dugaiczyk et al., Proc. Natl. Acad. Sci. USA 79:71 (1982). These genes may also be obtained by reisolation of the genes by the technique of Lawn et al., Dugaiczyk et al. or synthesized *in vitro* by a custom gene manufacturer such as British Biotechnology, Ltd. One possible method of obtaining a HSA gene would be to screen a human liver cDNA library with oligonucleotide probes or screen a human liver cDNA expression library with anti-HSA antisera to identify HSA expressing human liver cDNAs. One suitable HSA structural gene is provided in SEQ ID NO: 3. Once a structural gene for HSA is recovered, it may be necessary to further tailor the gene. Following the isolation of a HSA structural gene, the gene is inserted into a suitable *Pichia pastoris* vector such as a plasmid or linear site-specific integrative vector.

Plasmid type vectors have long been one of the basic elements employed in recombinant DNA technology. Plasmids are circular extra-chromosomal double-stranded DNA found in microorganisms. Plasmids have been found to occur in single or multiple copies per cell. Included in plasmid DNA is the information required for plasmid reproduction, e.g. an autonomous replication sequence such as those disclosed by James M. Clegg in U.S. Patent 4,837,148, issued June 6, 1989, incorporated herein by reference. The autonomous replication sequences disclosed by Clegg provide a suitable means for maintaining plasmids in *Pichia pastoris*. Additionally one or more means of phenotypically selecting the plasmid in transformed cells may also be included in the information encoded in the plasmid.

Suitable integrative vectors for the practice of the present invention are the linear site-specific integrative vectors described by James M. Clegg, in U.S. Patent 4,882,279, issued November 21, 1989, which is incorporated herein by reference. These vectors comprise a serially arranged sequence of at least 1) a first insertable DNA fragment; 2) a selectable marker gene; and 3) a second insertable DNA fragment. An expression cassette containing a heterologous structural gene is inserted in this vector between the first and second insertable DNA fragments either before or after the marker gene. Alternatively, an expression cassette can be formed *in situ* if a regulatory region or promoter is contained within one of the insertable

fragments to which the structural gene may be operably linked.

The first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. The various components of the integrative vector are serially arranged forming a linear fragment of DNA such that the expression cassette and the selectable marker gene are positioned between the 3' end of the first insertable DNA fragment and the 5' end of the second insertable DNA fragment. The first and second insertable DNA fragments are oriented with respect to one another in the serially arranged linear fragment as they are oriented in the parent genome.

Nucleotide sequences useful as the first and second insertable DNA fragments are nucleotide sequences which are homologous with separate portions of the native genomic site at which genomic modification is to occur. For example, if genomic modification is to occur at the locus of the alcohol oxidase gene, the first and second insertable DNA fragments employed would be homologous to separate portions of the alcohol oxidase gene locus. Examples of nucleotide sequences which could be used as first and second insertable DNA fragments are deoxyribonucleotide sequences selected from the group consisting of the *Pichia pastoris* alcohol oxidase (AOX1) gene, dihydroxyacetone synthase (DAS1) gene, p40 gene and HIS4 gene. The AOX1 gene, DAS1 gene, p40 gene and HIS4 genes are disclosed in U.S. Patents 4,855,231 and 4,885,242 both incorporated herein by reference. The designation DAS1 is equivalent to the DAS designation originally used in U.S. Patents 4,855,231 and 4,885,242.

The first insertable DNA fragment may contain an operable regulatory region which may comprise the regulatory region utilized in the expression cassette. The use of the first insertable DNA fragment as the regulatory region for an expression cassette is a preferred embodiment of this invention. Figure 1 provides a diagram of a vector utilizing the first insertable DNA fragment as a regulatory region for a cassette. Optionally, as shown in Figure 1, an insertion site or sites and a 3' termination sequence may be placed immediately 3' to the first insertable DNA fragment. This conformation of the linear site-specific integrative vector has the additional advantage of providing a ready site for insertion of a structural gene without necessitating the separate addition of a compatible 3' termination sequence.

If the first insertable DNA fragment does not contain a regulatory region, a suitable regulatory region will need to be inserted linked to the structural gene, in order to provide an operable expression cassette. Similarly, if no 3' termination sequence is provided at the insertion site to complete the expression cassette, a 3' termination sequence can be operably linked to the 3' end of the structural gene.

It is also highly desirable to include at least one selectable marker gene in the DNA used to transform the host strain. This facilitates selection and isolation of those organisms which have incorporated the transforming DNA. The marker gene confers a phenotypic trait to the transformed organism which the host did not have, e.g., restoration of the ability to produce a specific amino acid where the untransformed host strain has a defect in the specific amino acid biosynthetic pathway, or provides resistance to antibiotics and the like. Exemplary selectable marker genes may be selected from the group consisting of the HIS4 gene (disclosed in U.S. Patent 4,885,242) and the ARG4 gene (disclosed in U.S. Patent 4,818,700 incorporated herein by reference) from *Pichia pastoris* and *Saccharomyces cerevisiae*, the invertase gene (SUC2) (disclosed in U.S. Patent 4,857,467 incorporated herein by reference) from *Saccharomyces cerevisiae*, or the G418<sup>R</sup>/kanamycin resistance gene from the *E. coli* transposable elements Tn601 or Tn903.

Those skilled in the art recognize that additional DNA sequences can also be incorporated into the vectors employed in the practice of the present invention, such as, for example, bacterial plasmid DNA, bacteriophage DNA, and the like. Such sequences enable the amplification and maintenance of these vectors in bacterial hosts.

The insertion of the HSA structural gene into suitable vectors may be accomplished by any suitable technique which cleaves the chosen vector at an appropriate site or sites and results in at least one operable expression cassette containing the HSA structural gene being present in the vector. Ligation of the HSA structural gene may be accomplished by any appropriate ligation technique such as utilizing T4 DNA ligase.

The initial selection, propagation, and optional amplification of the ligation mixture of the HSA structural gene and a vector is preferably performed by transforming the mixture into a bacterial host such as *E. coli* - (although the ligation mixture could be transformed directly into a yeast host but, the transformation rate would be extremely low). Suitable transformation techniques for *E. coli* are well known in the art. Additionally, selection markers and bacterial origins of replication necessary for the maintenance of a vector in a bacterial host are also well known in the art. The isolation and/or purification of the desired plasmid containing the HSA structural gene in an expression system may be accomplished by any suitable means for the separation of plasmid DNA from the host DNA. Similarly the vectors formed by ligation may be tested, preferably after propagation, to verify the presence of the HSA gene and its operable linkage to a

regulatory region and a 3' termination sequence. This may be accomplished by a variety of techniques including but not limited to endonuclease digestion, gel electrophoresis, or Southern hybridization.

Transformation of plasmids or linear vectors into yeast hosts may be accomplished by suitable transformation techniques including but not limited to those taught by Clegg and Barringer, U.S. Patent 5 4,929,555; Hinnen et al., Proc. Natl. Acad. Sci. 75, (1978) 1929; Ito et al., J. Bacteriol. 153, (1983) 163; Clegg et al. Mol. Cell Biol. 5 (1985), pg. 3376; D. W. Stroman et al., U.S. Patent 4,879,231, issued November 7, 1989; or Sreekrishna et al., Gene, 59 (1987), pg. 115. Preferable for the practice of this invention is the transformation technique of Clegg et al. (1985). It is desirable for the practice of this invention to utilize linear vectors and select for insertions by Southern hybridization.

The yeast host for transformation may be any suitable methylotrophic yeast. Suitable methylotrophic yeasts include but are not limited to yeast capable of growth on methanol selected from the group consisting of the genera *Hansenula* and *Pichia*. A list of specific species which are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Presently preferred are methylotrophic yeasts of the genus *Pichia* such as the auxotrophic *Pichia pastoris* GS115 (NRRL Y-15851); *Pichia pastoris* GS190 (NRRL Y-18014) disclosed in U.S. Patent 4,818,700; and *Pichia pastoris* PPF1 (NRRL Y-18017) disclosed in U.S. Patent 4,812,405. Auxotrophic *Pichia pastoris* strains are also advantageous to the practice of this invention for their ease of selection. It is recognized that wild type *Pichia pastoris* strains (such as NRRL Y-11430 and NRRL Y-11431) may be employed with equal success if a suitable transforming marker gene is selected, such as the use of SUC2 to transform *Pichia pastoris* to a strain capable of growth on sucrose or an antibiotic resistance marker is employed, such as resistance to G418.

Transformed *Pichia pastoris* cells can be selected for by using appropriate techniques including but not limited to culturing previously auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype ("methanol slow"), or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformant.

Isolated transformed *Pichia pastoris* cells are cultured by appropriate fermentation techniques such as shake flask fermentation, high density fermentation or the technique disclosed by Clegg et al. in, High-Level Expression and Efficient Assembly of Hepatitis B Surface Antigen in the Methylotrophic Yeast, Pichia Pastoris 5 Bio/Technology 479 (1987). Isolates may be screened by assaying for HSA production to identify those isolates with the highest HSA production level.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors. For the large-scale production of recombinant DNA-based products in methylotrophic yeast, a three stage, high cell-density, batch fermentation system is normally the preferred fermentation protocol employed. In the first, or growth stage, expression hosts are cultured in defined minimal medium with an excess of a non-inducing carbon source (e.g. glycerol). When grown on such carbon sources, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. It is presently preferred, during this growth stage, that the pH of the medium be maintained at about 5. Next, a short period of non-inducing carbon source limitation growth is allowed to further increase cell mass and derepress the methanol responsive promoter. The pH of the medium during this limitation growth period is adjusted to the pH value to be maintained during the production phase, which is generally carried out at about pH 5 to about pH 6, preferably either about pH 5.0 or about pH 5.8. Subsequent to the period of growth under limiting conditions, methanol alone (referred to herein as "limited methanol fed-batch mode") or a limiting amount of non-inducing carbon source plus methanol (referred to herein as "mixed-feed fed-batch mode") are added in the fermentor, inducing the expression of the heterologous gene driven by a methanol responsive promoter. This third stage is the so-called production stage.

The invention will now be described in greater detail in the following non-limiting examples.

#### Examples

General information pertinent to the Examples:

#### Strains

55      *Pichia pastoris* GS115 (his 4) NRRL Y-15851  
*E. coli* JM103 delta (lac pro) thi rpsL (strA) supE endA sbcB hsdR.  
*E. coli* K12 MC1061 NRRL1-18016 (F<sup>-</sup>, araD139 delta (lac 1POZY)x74 galK galU hsr hsm(+)) rpsL delta (araABOIC leu)7697.

*E. coli* DG75' (hsd1, leu6, lacY, thr-1, supE44, tonA21, lambda<sup>-</sup>)

Buffers, Solutions and Media

5 The buffers, solutions, and media employed in the following examples have the compositions given below:

10	dH <sub>2</sub> O	deionized H <sub>2</sub> O that has been treated with a milli-Q (Millipore) reagent water system.
15	1M Tris buffer	121.1 g Tris base in 800 mL of H <sub>2</sub> O; adjust pH to the desired value by adding concentrated (35%) aqueous HCl; allow solution to cool to room temperature before final pH adjustment, dilute to a final volume of 1 L.
20	TE buffer	1.0 mM EDTA in 0.01 M (pH 8.0) Tris buffer
25	SED	1 M sorbitol 25 mM EDTA 50 mM DTT, added prior to use --adjust to pH 8
30	SCE	9.1 g sorbitol 1.47 g Sodium citrate 0.168 g EDTA --pH to 5.8 with HCl in 50 ml dH <sub>2</sub> O and autoclave
35	CaS	1 M sorbitol 10 mM CaCl <sub>2</sub> --filter sterilize
40	SOS:	1 M sorbitol 0.3x YPD
45	PEG	10 mM CaCl <sub>2</sub> 20% polyethylene glycol-3350 10 mM CaCl <sub>2</sub> 10 mM Tris-HCl (pH 7.4) --filter sterilize
50	Solution A	0.2 M Tris-HCl (pH 7.5) 0.1 M MgCl <sub>2</sub> 0.5 M NaCl 0.01 M dithiothreitol (DTT) 0.2 M Tris-HCl (pH 7.5)
55	Solution B	0.1 M MgCl <sub>2</sub> 0.1 M DTT 4 μl solution B 4 μl 10 mM dATP 4 μl 10 mM dTTP 4 μl 10 mM dGTP 4 μl 10 mM dCTP 4 μl 10 mM ATP 5 μl T <sub>4</sub> ligase (2 U/μl) 12 μl H <sub>2</sub> O
60	Solution C (keep on ice)	Recipe for Solution C was modified from Zoller & Smith
65	20X SSPE	4.4 g NaOH 7.4 g Na <sub>2</sub> EDTA 27.6 g NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O 210 g NaCl --pH adjusted to 7.5-8.0 with NaOH --H <sub>2</sub> O to 1 liter
70	50X Denhardt's	5 g Ficoll 400

		5 g Polyvinylpyrrolidine
5	20X SSC	5 g BSA Fraction V
		H <sub>2</sub> O to 500 ml
		175.3 g NaCl
		88.2 g sodium citrate
		--pH to 7.0 with NaOH
		--H <sub>2</sub> O to 1 liter
		5.0 g yeast extract
		10.0 g tryptone
		5.0 g NaCl
10	LB Broth, 1 liter	96.8 g Trizma Base
		9.74 g glycine
		water to 1 liter
	10X Transfer Buffer	500 mls 10X Transfer Buffer
15	Transfer Buffer for Tank	1000 mls methanol
		3500 mls water
	Western Buffer - for 1 liter	2.5 g gelatin put in solution by microwaving first in 100 mls water
20		100 mls 10X PBS
		1 ml 50% Tween-20
	Coating Buffer	4 mls 5% sodium azide
25		dH <sub>2</sub> O to 1 liter
		0.160 g Na <sub>2</sub> CO <sub>3</sub> (sodium carbonate)
	Tris Buffered Saline (TBS)	0.294 g NaHCO <sub>3</sub> (sodium carbonate)
30	Tris Buffered Saline/Tween (TBST)	Add distilled water to 100 ml. Do not pH. (pH should be 9.5)
		26.1 g NaCl
		2.63 g Tris
		Add distilled water to 3 liters.
		Adjust pH to 7.5 with HCl.
	Blotto Buffer	1 liter of TBS
35		2.5 ml of 20% Tween-20
		50 g of non-fat dry milk (Carnation)
		1 g thimerosal (Sigma)
		100 µl of antifoam (Sigma, 30% emulsion)
		2.5 ml of 20% Tween-20
		100 ml 10x PBS (house stock)
		Add distilled water to 1 liter
	Ligation Buffer	Adjust pH to 7.5
40		50 mM Tris-HCl (pH 7.4)
		10 mM MgCl <sub>2</sub>
		10 mM dithiothreitol
	Phosphatase Buffer	1 mM ATP
45		50 mM Tris-HCl (pH 9.0)
		1 mM MgCl <sub>2</sub>
		1 mM ZnCl <sub>2</sub>
	Bsu36I buffer	1 mM spermidine
50		100 mM NaCl
		10 mM Tris-HCl (pH 7.4)
		10 mM MgCl <sub>2</sub>
	Csp45I buffer	100 µg/ml BSA
		60 mM NaCl
		10 mM Tris-HCl, pH 7.5
55	RReact 1 buffer	7 mM MgCl <sub>2</sub>
		100 µg/ml BSA
		50 mM Tris-HCl, pH 8.0
		10 mM MgCl <sub>2</sub>
		100 µg/ml BSA

	REact 2 buffer REact 3 buffer HS buffer	REact 1 buffer + 50 mM NaCl REact 1 buffer + 100 mM NaCl 50 mM Tris-HCl, pH 7.5 10 mM MgCl <sub>2</sub> 100 mM NaCl 1 mM DTT 100 µg/ml BSA
5		42 mls Phosphoric Acid, 85% 1.8 g Calcium Sulfate • 2H <sub>2</sub> O 28.6 g Potassium Sulfate 23.4 g Magnesium Sulfate • 7H <sub>2</sub> O 6.5 g Potassium Hydroxide
10	10X Basal Salts	
15	Ptm <sub>1</sub> Trace Salts Solution	6.0 g Cupric Sulfate • 5H <sub>2</sub> O 0.08 g Sodium Iodide 3.0 g Manganese Sulfate • H <sub>2</sub> O 0.2 g Sodium Molybdate • H <sub>2</sub> O 0.02 g Boric Acid 0.5 g Cobalt Chloride 20.0 g Zinc Chloride 65.0 g Ferrous Sulfate • H <sub>2</sub> O 0.20 g Biotin 5.0 mls Sulfuric Acid
20	YPD (yeast extract peptone dextrose medium)	10 g bacto yeast extract 20 g peptone 10 g dextrose water to 1 liter
25		13.4 g yeast nitrogen base with ammonium sulfate, and without amino acids 400 µg biotin 10 ml glycerol water to 1 liter
30	MGY (minimal glycerol medium)	
35	MM (minimal methanol medium)	Same as MGY, except that 5 ml methanol is used in the place of 10 ml glycerol.  13.4 g yeast nitrogen base with ammonium sulfate and without amino acids 400 µg biotin 182 g sorbitol 10 g glucose 2 g Histidine assay mix (Gibco) 50 mg glutamine 50 mg methionine 50 mg lysine 50 mg leucine 50 mg isoleucine 10 g agarose water to 1 liter
40	SDR (supplemented dextrose regeneration medium):	
45		100 ml/liter Potassium phosphate buffer, (pH 6.0) 13.4 grams/liter Yeast nitrogen base with ammonium sulfate 400 µg/liter biotin 10 ml/liter glycerol
50	BMGR (Buffered minimal glycerol-enriched medium)	
55		

	Amino acids	glutamic acid, methionine, lysine, leucine and isoleucine: each at 5 mg/liter; all the other amino acids except histidine at 1 mg/liter
5	Nucleotides	adenine sulfate, guanine hydrochloride, uracil, and xanthine, each at 40 µg/liter
10	Vitamins	thiamine hydrochloride, riboflavin, and calcium pantothenate, each at 2 µg/liter; pyridoxide hydrochloride and nicotinic acid, each at 4 µg/liter; pyridoxamine hydrochloride and pyridoxal hy- drochloride, each at 1 µg/liter; para-amino benzoic acid at 0.3 µg/liter; folic acid at 0.03 µg/liter
15	Trace minerals	magnesium sulfate at 800 µg/liter; ferrous sulfate at 40 µg/liter; manganese sulfate at 80 µg/liter; sodium chloride at 40 µg/liter
20	BMGY (Buffered minimal glycerol-complex medium)	100 ml/liter potassium phosphate buffer, (pH 6.0) 13.4 grams/liter yeast nitrogen base with am- monium sulfate and without amino acids biotin at 400 µg/liter glycerol at 10 ml/liter yeast extract at 10 g/liter peptone at 20 g/liter
25	BMMR (Buffered minimal methanol-enriched medium)	Same as BMGR, with the exception that 5 ml methanol/liter is added in the place of glycerol
30	BMMY (Buffered minimal methanol -complex medium)	Same as BMGY, with the exception that 5 ml methanol/liter is added in the place of glycerol
35		

### Techniques

40 Suitable techniques for recombinant DNA lab work may be found in many different references including  
but not limited to: Methods in Enzymology, (Orlando, FL: Academic Press, Inc.), particularly Volume 152,  
published as, Guide to Molecular Cloning Techniques, by Berger and Kimmel (Orlando, FL: Academic  
Press, Inc., 1987) and Molecular Cloning/A Laboratory Manual, by Sambrook et al., 2d ed. (Cold Spring  
Harbor Laboratory Press, 1989) and which are all hereby incorporated by reference.

### 45 Example I

#### Construction of mHSA13

##### 50 Mutagenesis of HSA Structural Gene Insert

DNA encoding HSA was obtained from pHSA13, disclosed in European Patent Application 0 344 459,  
herein incorporated by reference, by EcoRI digestion. A 2069 bp fragment was recovered by elec-  
trophoresis on a 1% agarose gel. The DNA was mutagenized by the following procedure to make the  
following changes: 1) an EcoRI restriction site was added immediately prior to the ATG of the HSA signal  
55 sequence, and 2) an EcoRI restriction site was added immediately adjacent to the TAA stop codon in the  
HSA cDNA.

The oligonucleotides employed in the mutagenesis were:

1) 5' mutagenesis to add EcoRI site, mutagenizing nucleotide sequence:

5' CCC TCA CAC GCC TTT GAA TTC ATG AAG TGG GTA ACC 3' (SEQ ID NO:4)

2) 3' mutagenesis to add EcoRI site, mutagenizing nucleotide sequence:

5' GCC TTA GGC TTA TAA GAA TTC AGT TTA AAA GCA TCT CAG 3' (SEQ ID NO:5)

and were synthesized using an Applied Biosystems DNA Synthesizer, Model 380A using cyanoethyl-phosphoramidite chemistry.

1.2 µg of double-stranded m13mp10 were digested with EcoRI and dephosphorylated and ligated with 450 ng of the previously isolated 2069 bp fragment containing the HSA structural gene.

The ligation mixture was transformed into competent JM103 cells (competent JM103 were prepared as described in Example II for MC1061 cells). The mixture was then plated on LB media containing IPTG and X-gal and the plates screened for clear plaques. DNA was recovered from transformants and digested with Hind III. The correct phage demonstrated bands of 7369 and 1950 bp and was called mHSA13.

A. A large scale miniprep was performed on positive plaques which had been incubated for approximately 7 hours in 2 mls of L media. 25 mls of LB media was inoculated with 250 µl of freshly grown JM103 cells. The culture was grown for 1 hour and inoculated with 100 µl of the 7 hour old plaque culture. The culture was then grown overnight. The culture was centrifuged twice at 10,000 rpm for 10 minutes on a Sorvall RC-5B rotor SS34 to clear the supernatant. 3.5 ml of 20% PEG/2.5 M NaCl was added to the culture and it was incubated for 5 hours at 4°C. The culture was then centrifuged again as above for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2 mls of TE buffer. The pellet was then extracted with phenol, equilibrated with TE, extracted with phenol/chloroform, extracted twice with CHCl<sub>3</sub> and once with ether. 8 M LiCl was added to attain a final concentration of 0.8 M. 3 volumes of ethanol were added and the solution left overnight at -20°C to precipitate the DNA present. The solution was next centrifuged for 10,000 rpm for 10 minutes as previously described and rinsed with 70% ethanol. The precipitate was resuspended in 150 µl of 10 mM Tris (pH 7.4).

B. One pmole of M13 recombinant template was mixed with 20 pmole of oligonucleotide 1 (for 5' mutagenesis to create an EcoRI site), 1 µl of solution A and dH<sub>2</sub>O was added to give a final volume of 10 µl. The sample was incubated at 65°C for 5 minutes, and the temperature was then reduced to 37°C for 30 minutes.

C. The following was then added to the sample:

30	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="padding: 2px;">Solution B</td><td style="padding: 2px;">1 µl</td></tr> <tr> <td style="padding: 2px;">10 mM dATP</td><td style="padding: 2px;">1 µl</td></tr> <tr> <td style="padding: 2px;">10 mM dCTP</td><td style="padding: 2px;">1 µl</td></tr> <tr> <td style="padding: 2px;">10 mM dGTP</td><td style="padding: 2px;">1 µl</td></tr> <tr> <td style="padding: 2px;">10 mM dTTP</td><td style="padding: 2px;">1 µl</td></tr> <tr> <td style="padding: 2px;">5 u/µl Klenow</td><td style="padding: 2px;">2 µl</td></tr> <tr> <td style="padding: 2px;">dH<sub>2</sub>O</td><td style="padding: 2px;">3 µl</td></tr> <tr> <td style="padding: 2px;"></td><td style="padding: 2px;">20 µl</td></tr> </table>	Solution B	1 µl	10 mM dATP	1 µl	10 mM dCTP	1 µl	10 mM dGTP	1 µl	10 mM dTTP	1 µl	5 u/µl Klenow	2 µl	dH <sub>2</sub> O	3 µl		20 µl
Solution B	1 µl																
10 mM dATP	1 µl																
10 mM dCTP	1 µl																
10 mM dGTP	1 µl																
10 mM dTTP	1 µl																
5 u/µl Klenow	2 µl																
dH <sub>2</sub> O	3 µl																
	20 µl																
35																	

40 and allowed to incubate at 15°C for at least 4-6 hours.

D. The sample was then diluted 1:40 with dH<sub>2</sub>O. 5 µl was used to transform 6 tubes of competent JM103 cells (200 µl each). The transformed JM103 cells were plated on rich media in a soft agar overlay.

E. The positive plaques were then screened for by filter hybridization.

45 A hybridization probe of 15 pmole of complementary oligonucleotide in a total volume of 25 µl total volume was heated to 65°C for 10 minutes. 3 µl 10X kinase buffer (Maniatis), 1 µl  $\gamma$ -ATP and 1 µl polynucleotide kinase (100 u/µl) were added to the sample. The sample was incubated for 1 hour at 37°C and run through G-50 fine Sephadex. The first peak off the column was collected.

50 Nitrocellulose filters were prepared for hybridization with the above probe by placing and orienting the filters on the transformation plates for 5-10 minutes. The filters were then removed from the plates and floated on a denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 3 minutes with the backside on top of the solution. The filters were then submerged in the denaturing solution for 5 minutes. The nitrocellulose filters were transferred to a neutralizing solution (1 M Tris/HCl, pH 8; 1.5 M NaCl) for 5 minutes. The neutralized filter was then transferred to 2XSSC (1XSSC is 150 mM NaCl, 15 mM NaCitrate) for 5 minutes. The filter was then air dried and baked for 1 hour at 80°C under a vacuum. The filters were prehybridized for 1 hour at 65°C in a sealed plastic bag containing 5 ml of hybridization buffer filter, 10X Denhardts (1X Denhardts is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin) 0.5% SDS and 5XSSPE. The hybridization buffer was replaced with 5 ml/filter of fresh hybridization

buffer. The screening oligonucleotides utilized were as follows:

- 1) 5' mutagenesis the screening oligonucleotide was  
5' GCC TGG GAA TTC ATG AAG 3' (SEQ ID NO:6)
- 2) 3' mutagenesis the screening oligonucleotide was  
5' TTA TAA GAA TTC AGT TTA 3' (SEQ ID NO:7)

The previously prepared screening oligonucleotide was first incubated at 65°C for 5 minutes, and then enough probe was added to the fresh hybridization buffer containing the filter to give 1X10<sup>6</sup> cpm/ml. Hybridization was performed at 5°C below the calculated melting temperature of the probe for 4 hours.

The filters were then washed three times for 10 minutes each with 6XSSC at room temperature. The filters were finally washed one time with 6XSSC at the hybridization temperature. The filters were placed on a 3 MM Whatman paper to dry, and then exposed to film (marked for orientation) overnight.

Three positive plaques were each picked and grown separately in 2 mls of LB broth at 37°C for 5 hours.

F. Mini template preps were performed on each of these positive plaques.

One ml of the plaque culture was transferred into an Eppendorf tube and centrifuged for 5 minutes in a Eppendorf Model 5414 Centrifuge. 800 µl of the supernatant was recovered and 200 µl of 20% PEG with 2.5M NaCl was added thereto. The supernatant was incubated at room temperature for 10 minutes. The supernatant was centrifuged for 10 minutes in the Eppendorf centrifuge previously used. The supernatant was removed by aspiration and the pellet formed by centrifuging was redissolved in 200 µl TE (10 mM Tris, pH 7.4; 1 mM EDTA). The redissolved pellet was then phenol/chloroform extracted and the template DNA in the upper aqueous phase was precipitated by the addition of a LiCl solution until a 0.8 M concentration was reached. To the solution was added 2 1/2-3 volumes of ethanol and precipitated on dry ice for 5 minutes. The precipitate was centrifuged for 10 minutes in the previously mentioned Eppendorf centrifuge. The final volume was brought up to 150 µl with TE.

G. 200 µl of competent JM103 cells were transformed with the recovered DNA. 1 µl and 1 µl of a 1/10 dilution of the isolated phase DNA was used in the transformation.

H. The transformation mixture was plated and plaques were screened with oligonucleotides as previously described in step E.

I. A large scale miniprep was performed on positive plaques which had been incubated for approximately 7 hours in 2 mls of L media. 25 mls of LB media was inoculated with 250 µl of freshly grown JM103 cells. The culture was grown for 1 hour and inoculated with 100 µl of the 7 hour old plaque culture. The culture was then grown overnight. The culture was then centrifuged twice at 10,000 rpm for 10 minutes on a Sorvall RC-5B rotor SS34 to clear the supernatant. 3.5 ml of 20% PEG/2.5M NaCl was added to the culture and it was incubated for 5 hours at 4°C. The culture was then centrifuged again as above for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2 mls of TE buffer. The pellet was then extracted with phenol, equilibrated with TE, extracted with phenol/chloroform, extracted twice with CHCl<sub>3</sub> and once with ether. 8 M LiCl was added to attain a final concentration of 0.8 M LiCl. 3 volumes of ethanol were added and the solution left overnight to precipitate the DNA present. The solution was next centrifuged for 10,000 rpm for 10 minutes as previously described and rinsed with 70% ethanol. The precipitate was resuspended in 150 µl of 10mM Tris (pH 7.4).

J. The positive plaques were then sequenced by dideoxy sequencing to find the M13 constructs with the correct mutations.

K. Repeat steps B-J using M13 constructs with the correct 5' mutations as templates and the second oligonucleotide as a primer for 3' mutagenesis. The correct mutation was designated mHSA140.

L. Recover RF DNA of mHSA140 using the alkaline lysis method of Maniatis.

#### Example II

##### Construction of the pHSA140 Expression Vectors

pA0804 is available in an *E. coli* host from the Northern Regional Research Center of the United States Department of Agriculture, Peoria, Illinois, accession number B-18114. pA0804 is recovered by isolating the plasmid DNA, digesting with EcoRI, gel electrophoresing to recover the ~7.5 kb fragment, which is linear pA0804 cut at its unique EcoRI site.

pA0804 is a vector capable of site-specific disruption of the *Pichia pastoris* AOX1 locus. It contains the following elements: the AOX1 promoter and transcription terminator separated by a unique EcoRI cloning site; the wild-type *Pichia* HIS4 gene; a genomic segment of DNA from the 3' end of the AOX1 locus downstream of the transcription terminator; and sequences necessary for selection and replication in a

bacterial host. The components are arranged such that a BgIII restriction digest of the plasmid releases a DNA fragment containing the expression cassette and selective marker whose ends are homologous to a continuous portion of the genome, the AOX1 locus, and can be stably inserted into the chromosome during transformation. Additionally the ampicillin resistance gene and the ori from plasmid pBR322 are also contained in the pA0804 plasmid.

A vector containing the gene coding for the production of HSA was constructed from pA0804 and mHSA140. pA0804 was digested with EcoRI and the ends were dephosphorylated by treatment with alkaline phosphatase (1 U enzyme at 37°C for 1 hr. in 50 mM Tris•Cl, pH 9.0, 1 mM MgCl<sub>2</sub>, 100 mM ZnCl<sub>2</sub>, 1 mM spermidine). mHSA140 was also digested with EcoRI, and a 1829 bp fragment encoding HSA was released. This fragment was purified using 0.8% preparative agarose gel electrophoresis. 60 ng of the fragment were ligated to 240 ng of pA0804 by incubation at 23°C for 1 hr in 66 mM Tris•Cl, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1 mM ATP, with 1 Weiss Unit of T4 ligase in a 10 µl reaction volume. The ligation reaction was used to transform competent MC1061 cells to ampicillin resistance.

MC1061 was rendered competent for transformation in the following manner. A mid-log culture (50 ml) of *E. coli* MC1061 was harvested by centrifugation in an IEC DPR 600 clinical centrifuge at 3,000 rpm for 5 min at 4°C and washed in 10 mM NaCl. The culture was resuspended in 25 ml of 50 mM CaCl<sub>2</sub> for 30 min at 0°C. The cells were centrifuged as above and resuspended in 2 ml of 50 mM CaCl<sub>2</sub>.

For transformation, the ligation reaction was added to 200 µl of the competent cell suspension and incubated at 0°C on ice for 15 minutes, heat shocked at 37°C for 5 minutes and incubated at 23°C for 5 minutes. The cells were plated directly onto LB agar plates containing 50 µg/ml ampicillin. The plates were incubated at 37°C for 10-16 hours. The resulting colonies were Amp<sup>R</sup>. The resistant colonies were harvested and characterized by restriction digestion. Cells were grown in 5 ml of L-broth containing 50 µg/ml ampicillin for 5 hr at 37°C and DNA was prepared by the method of Birnboim and Doly [Nucleic Acids Research 7:1513 (1979)]. The minipreps displaying 4750, 3000 and 1900 bp fragments upon PvuII digestion were chosen and designated pHSA140.

### Example III

#### Transformation of *Pichia pastoris*

*Pichia pastoris* strains containing the vectors described in Example II were generated in the following manner. Methanol utilization deficient (Mut<sup>-</sup> or methanol slow) and wild type methanol utilization (Mut<sup>+</sup> or metaanol normal) strains were developed.

*Pichia pastoris* strain GS115 (his4; NRRL Y-15851) was transformed using the spheroplast transformation technique described by Cregg et al., Bio/Technology 5:479-485 (1987). See also U.S. 4,879,231.

#### A. Mut<sup>+</sup> Strains

To direct integration of the vector to the AOX1 locus, 2 and 10 µg of SacI- digested pHSA140 were separately transformed into 5 of OD<sub>600</sub> (or 25 × 10<sup>7</sup> cells) of GS115. Transformants were regenerated on minimal media and screened for the His<sup>+</sup> phenotype. Several His<sup>+</sup> transformants were then screened by Southern analysis for the site of integration and vector copy number (Example V).

#### B. Mut<sup>-</sup> Strains

To develop Mut<sup>-</sup> strains, in which the HSA expression cassette integrates into and disrupts the AOX1 structural gene, vector pHSA140 was digested with PvuI and then partially digested with BgIII. The digest was then size fractionated on a 0.8% agarose gel and DNA in the size range of 6.0-9.0 kb was isolated (the expression cassette was expected to be ~ 7.4 kb). 5µg of this DNA were used to transform 5 OD<sub>600</sub> (25 × 10<sup>7</sup> cells) of GS115 by the spheroplast method. His<sup>+</sup> cells were identified and then screened for the Mut<sup>-</sup> phenotype as follows.

Transformants were pooled by scraping the surface of the plate in the presence of sterile distilled water and sonicated at low output for 15 seconds. They were subsequently diluted to an A<sub>600</sub> = 0.1 and plated at dilutions of 10<sup>-3</sup> and 10<sup>-4</sup>, in duplicate onto minimal plates containing glycerol as the carbon source, and incubated at 30°C for 2-3 days. They were then replica-plated onto minimal plates to which 100 µl of methanol was added in the vapor phase. After a 24-hour incubation at 30°C, it was apparent that 4% of the transformants were growing more slowly on methanol than the rest of the transformants. Five of the His<sup>+</sup> Mut<sup>-</sup> isolates were examined by Southern analysis (Example V).

Example IVYeast DNA Miniprep

5       $10^4$  cells/ml were seeded in 5 ml YPD at 30°C overnight and then pelleted using a Damon IEC DPR600 clinical centrifuge at 3,000 rpm for 5 minutes. The pellet was resuspended in 0.5 ml of 1 M sorbitol, 0.1 ml 0.5 M EDTA, pH 8 and the sample transferred to a 1.5 ml microfuge tube. 0.02 ml of 2.5 mg/ml Zymolyase 100,000 (Miles Laboratories) was added, and the sample was incubated at 37°C for 60 minutes. The cells were pelleted using the microfuge for 1 minute at high speed, and resuspended in 0.5 ml of 50 mM Tris·Cl, pH 7.4 and 20 mM EDTA. 0.05 ml of 10% SDS was added, the sample mixed, and incubated at 65°C for 30 minutes. 0.2 ml of 5 M potassium acetate, pH 5.2, was added and the sample was incubated on ice for 60 minutes. The sample was again spun in a microfuge at high speed for 5 minutes.

The supernatant was transferred to a fresh 1.5 ml microfuge tube and 1 volume of isopropanol at room temperature was added. The sample was mixed and allowed to sit at room temperature for 5 minutes, then 15 spun very briefly (10 seconds) in a microfuge at high speed. The supernatant was poured off and the pellet air dried. After resuspending the pellet in 0.3 ml of 10 mM Tris·Cl, pH 7.4 and 1 mM EDTA, 15 µl of a 1 mg/ml solution of pancreatic RNase was added, and the sample was incubated at 37°C for 30 minutes. 0.03 ml of 3 M sodium acetate was added, the sample mixed, and 0.2 ml of isopropanol added. The sample was spun in a microfuge at high speed to pellet the DNA. The supernatant was then poured off, the pellet dried and resuspended in 0.1-0.3 ml of 10 mM Tris·Cl, pH 7.4 and 1 mM EDTA. (Note: Before using the DNA in a restriction digest, it may be necessary to spin the solution for 15 minutes at high speed in the microfuge to remove any insoluble material which may inhibit the digestion).

Example VStrain Characterization

25     DNA was prepared from the transformed *Pichia* cells (Example III) and from untransformed host *Pichia* cells as described in Example IV, and digested with EcoRI. The samples were electrophoresed on 0.8% agarose gels, and Southern blots were performed (Maniatis et al, 1982). The filters were hybridized with an AOX1 specific probe or with a HIS4 specific probe to determine where integration had occurred. The site of 30 integration was determined by comparing the spectrum of hybridization of a given transformant with the wild type strain. Any alteration in the size of the wild type band was evidence of integration at that locus. A summary of the Southern hybridizations and strain characterization for the strains chosen for further 35 analysis is below.

Table I

40	Strain Name	Site of Integration	Vector Copy Number
	G + HSA140S1	AOX1	one
	G + HSA140S4	AOX1	two
	G + HSA140S3	AOX1	>two
45	G-HSA140S1	AOX1	one

Fermentor Growth of HSA-Expressing *Pichia* Strains

50     Inocula were prepared from selective plates and grown overnight at 30°C in buffered YNB containing 2% glycerol to an OD<sub>600</sub> of 0.5-10.0. An aliquot of 5-50 ml of the overnight culture was added to a 2-liter capacity fermentor, and the repressed growth phase continued in 5X basal salts containing 5 mM/L of PTM<sub>1</sub> salts at 30°C. The pH was maintained at 5.0 by the addition of 40% (v/v) ammonium hydroxide, and foaming was controlled by the addition of 5% (v/v) Struktol antifoam. Dissolved oxygen was maintained above 20% by increased aeration and agitation as needed. The temperature was maintained at about 30°C. 55 This batch growth phase continued for 20-30 hours until the glycerol was exhausted. The fermentation was then continued in either a methanol-limited fed-batch mode for Mut<sup>+</sup> strains or a methanol-excess fed-batch mode for Mut<sup>-</sup> strains.

a. Mut<sup>+</sup> fermentation; methanol-limited fed batch

Run 544: G + HSA140S1 (1 copy)

Run 557: G + HSA140S4 (2 copy)

5 Run 545: G + HSA140S3 (&gt;2 copy)

In order to continue building cell density as well as to prevent the accumulation of excess ethanol in the fermentor, the AOX1 promoter was derepressed to allow the expression of a small amount of alcohol oxidase before induction by the addition of methanol. This derepression was achieved by growth under glycerol-limited conditions at about pH 5.0. Following exhaustion of the glycerol in the initial growth phase, a 10 50% (w/v) glycerol feed (containing 12 ml/L of PTM<sub>1</sub> trace salts) was initiated at a rate of 8-16 ml/hour and continued until approximately 120-140 ml had been added. Full expression of the AOX1 promoter was then induced by the initiation of a methanol feed (100% MeOH plus 12 ml/L PTM<sub>1</sub> trace salts) at 1 ml/hour. The methanol feed was maintained for several hours until the culture responded to methanol limitation. This response was expressed as a sudden rise in dissolved oxygen upon a brief cessation of the methanol feed. 15 The methanol feed was then increased over an 8-12 hour period until a rate of 5.5 ml/hour was achieved. Fermentation was continued under these conditions for 82, 96 or 98 hours on methanol before the culture was harvested.

b. Mut<sup>-</sup> fermentation; methanol excess fed batch

20 The Mut<sup>-</sup> fermentations were conducted as described for the Mut<sup>+</sup> fermentations, except the MeOH feed was increased after 4 hours of 1 ml/hr feed to 3-4 ml/hr, to give a residual methanol concentration less than 0.5%.

25 Quantification of HSA Secreted into Growth Mediaa. ELISA

The ELISA procedure for human serum albumin requires the following reagents: Human Albumin (obtained 30 from Cappell, Organon Teknika), Goat anti-HSA antibody (obtained from Atlantic Antibodies), Goat anti-HSA antibody, peroxidase conjugated (obtained from Cappell, Organon Tecknika), and O-phenylenediamine (OPD), dichloride salt (obtained from Sigma, 10 mg/tablet). The HSA was reconstituted following the manufacturers directions. In this case (lot #26706) 3.0 ml of distilled water was added to the contents of the vial (the final concentration was 18.8 mg/ml). 29 aliquots of 100 µl each were labeled and quickfrozen.

35 16 aliquots were formed by diluting 100 µl of 18.8 mg/ml with 1.780 ml PBS (final 1.0 mg/ml). The 16 aliquots of 100 µl were labeled and quickfrozen.

100 more aliquots were formed by diluting 100 µl of 1 mg/ml with 9.9 ml PBS (final 10 µl). The 100 aliquots of 100 µl each, were then labeled and quickfrozen. This dilution was used to begin the standard curve of dilutions. The goat anti-HSA was supplied in solution. The goat anti-HSA reagent was divided into 50 µl 40 aliquots, labeled and quickfrozen. Goat anti-HSA conjugated to peroxidase was reconstituted by the addition of 2.0 mls of distilled water (final concentration 23 mg/ml). 50 µl of aliquots of the goat anti-HSA conjugate were then labeled and quickfrozen.

ELISA PROCEDURE:

45

Note: Use buffers at room temperatures only.

1. Make up coating buffer immediately before use. Dilute goat anti-HSA antibody 1:500. Add 200 µl of this solution to each well. Parafilm tightly and incubate one hour at 37° C.

2. Sharply flick contents of plate into sink. Wash 3 times with TBST. Wash 2 times with distilled water.

50 3. Add 200 µl blotto buffer to all wells. Parafilm tightly. Incubate overnight at 37° C.

4. Next morning, flick contents of wells into sink. Wash 3 times with TBST. Wash 2 times with distilled water.

5. Add 100 µl of TBST to all wells.

6. Dilute stock 10 µg/ml HSA standard with TBST.

55 S = stock from freezer = 10 µg/ml

SS = substock = 1:100 of S = 10,000 pg/100 µl

S<sup>-1</sup> = 1:10 of SS - 1,000 pg/100 µl

Dilute SS, 1:1 = 5,000 pg/100 µl

Dilute SS, 1:3.3 = 3,000 pg/100 µl

Dilute SS, 1:5 = 2,000 pg/100 µl

Dilute S<sup>-1</sup>, 1:1 = 500 pg/100 µl

Dilute S<sup>-1</sup>, 1:5 = 200 pg/100 µl

5 Dilute 3,000 pg/100 µl 1:1 = 1,500 pg/100 µl

8. Add 100 µl sample dilutions and standard curve dilutions to each well.

9. Parafilm tightly and incubate 2 hours at 37 °C.

10. Wash five times with TBST. Wash two times with distilled water.

11. Dilute goat anti-HSA conjugate 1:2000 with blotto buffer. Incubate for two hours at room temperature in the dark.

12. Wash three times with TBST. Wash two times with distilled water.

13. Immediately before use: add one pellet of OPD to 3 mls of distilled water in a dark container. Pipet 21 mls of water into a 50 ml Falcon tube. Add 3 mls OPD solution to the Falcon tube, add 10 µl of 30% H<sub>2</sub>O<sub>2</sub>, and mix.

14. Add 200 µl of this solution to each well. Parafilm tightly and incubate 10 minutes in the dark. Stop the reaction by addition of 50 µl 4.5 M sulfuric acid.

15. Read on ELISA reader at 492 nm using filter 4.

#### b. Data

20

The level of HSA secreted from each of the strains, and other information pertinent to the fermentations, is provided in Table II:

Table II

25

Fermentation of HSA Strains						
Run	Strain	Copy Number	Integration Site	Hours on MeOH	Cell Density (Wet) g/l	HSA in Broth g/l
30	537 G-HSA140S1	1	AOX1/Mut <sup>-</sup>	98	445	0.971
	544 G + HSA140S1	1	AOX1/Mut <sup>*</sup>	96	415	0.964
	557 G + HSA140S4	2	AOX1/Mut <sup>*</sup>	82	450	0.754
	545 G + HSA140S3	>2	AOX1/Mut <sup>*</sup>	96	353	0.185

35

#### Characterization of Recombinant Product

##### a. Gel analysis

40

Samples of fermentation broths of fermentation runs 537, 544 and 545 were withdrawn at different time points during the induction phase and analyzed by SDS gels and Coomassie blue staining. 5 µl of a 10-fold dilution of fermentor broth (equivalent to 0.5 µl) were applied to the gel. The relative intensity of the stained bands of rHSA (recombinant HSA) and the HSA standard confirmed the high (gram/liter) rHSA levels found by ELISA. In addition, the rHSA from all three fermentations and at all time points analyzed showed identical mobility with the HSA standard (69 Rd). The fact that the rHSA was the major protein species secreted by all HSA expression strains suggests high initial purity (>90%) of the rHSA secreted into the growth medium. Another protein species which migrates at approximately 45 Kd could also be detected in increasing intensity with respect to fermentation time. This protein species may be produced in a secondary processing event during secretion or as a proteolytic product post-secretion by proteases secreted into the fermentation broth, or both. It is related to rHSA, as it was detectable on Western blots by HSA specific polyclonal antisera.

##### b. N-terminal sequence

55

Protein sequence of the N-terminal region of secreted rHSA was obtained on a dialysed sample of fermentor broth. The sequence was determined on an Applied Biosystems Model 470A protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified by high performance liquid

chromatography with an Applied Biosystems Model 120A analyzer. The results showed that the rHSA N-terminus is aspartic acid, consistent with the N-terminal amino acid of HSA. There does not appear to be any other precursor type of rHSA. The balance of the sequence determined was identical to the known sequence for HSA.

5

#### Example VI

##### Construction of HSA expression vector pHSA313

10 The pHSA313 vector was constructed to provide a vector with an exact linkage between the 3' end of the native AOX1 5'regulatory region (promoter) and the start codon of the HSA structural gene.

##### A. Creation of pHSA113bC1a

15 About 200 ng of pHSA113 (disclosed in European Patent Application 0 344 459 and shown in Figure 7) was digested at 37°C for 1 hour with 1 unit of ClaI in 20 µl of REact 1 buffer. The digestion mixture was brought to 100 µl with water and extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 V/V), followed by extracting the aqueous layer with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by adjusting the NaCl concentration to 0.2 M and adding 3 volumes of cold ethanol. The mixture was allowed to stand on ice (4°C) for 10 minutes and the DNA precipitate was collected by centrifugation for 30 minutes at 10,000 x g in a microfuge at 4°C. The DNA pellet was washed 2 times with 70% aqueous cold ethanol. The washed pellet was vacuum dried and dissolved in 10 µl water to which 2 µl of 10 x ligation buffer, 2 µl of 1 mg/ml BSA, 6 µl of water and 1 unit T<sub>4</sub> DNA ligase were added. The mixture was incubated overnight at 4°C and a 10 µl aliquot was used to transform E. coli DG75' (Maniatis, et al.) to obtain pHSA113 $\Delta$ Cla, which represents the deletion of HIS4 and 3'AOX1, along with small stretches of pBR322 sequences used to link these sequences. The deletion of the HIS4, 3'AOX1 and pBR322 sequences removes one of two Csp45I sites present in the pHSA113 vector. The remaining Csp45I site is in the AOX1 5'regulatory region (promoter).

30 B. Creation of pXHSA113 $\Delta$ Cla

Digest 5 µg of pHSA113 $\Delta$ Cla for 1 hour at 37°C with 10 units of BstEII in 100 µl of REact 2 buffer. The digestion mixture was extracted with phenol and precipitated as detailed in step A. The DNA precipitate was dissolved in 100 µl of Csp45I buffer and digested at 37°C for 2 hours in the presence of 10 units of Csp45I. 35 The digested DNA was then phenol extracted and precipitated as described in step A. The DNA precipitate was dissolved in 20 µl of water and 10 µl aliquots were loaded on 2 neighboring wells of a 0.9% agarose gel. Following electrophoresis, the gel portion corresponding to one of the lanes was stained and this was used to locate the position of the Csp45I-BstEII fragment of pHSA113 $\Delta$ Cla in the unstained lane. The gel portion containing the larger Csp45I-BstEII fragment of pHSA113 $\Delta$ Cla was excised from the gel. The gel 40 portion containing the larger Csp45I-BstEII fragment was electroeluted into 500 µl of 5 mM EDTA, pH 8.0. The DNA solution was phenol extracted as detailed in step A and the DNA precipitate was dissolved in 100 µl water. The larger Csp45I-BstEII fragment was then ligated with the BstEII-Csp45I oligonucleotide linker described below. An aliquot (10 µl) of the Csp45I-BstEII fragments was ligated overnight at 4°C with 20 ng of annealed linker oligonucleotides 5'-CGAACG ATG AAG TGG (SEQ ID NO:8) and 5'-GTTACCCACT-45 TCATCGTTT (SEQ ID NO:9) in 20 µl ligase buffer containing 100 µg/ml BSA and 1 unit of T<sub>4</sub> DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pXHSA113 $\Delta$ Cla. The pXHSA113 $\Delta$ Cla vector by virtue of the linker described above has an exact linkage between the 3' end of the native AOX1 5'regulatory region (promoter) and the HSA ATG start codon with no extraneous DNA sequences.

50 C. Creation of pHSA313

1 µg of pXHSA113 $\Delta$ Cla was digested for 4 hours at 37°C with ClaI in 100 µl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 µl reaction volume for 30 minutes at 37°C. 55 Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/µl as described in step A and stored at -20°C.

1 µg of pA0807N (Figure 8, construction of which is described in European Patent Application 0 344 459) was digested for 4 hours at 37°C with PstI in 100 µl of REact 2 buffer. The digested DNA was

adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200  $\mu$ l reaction volume for 15 minutes at 55°C. At the end of 15 minutes another 10 units of phosphatase was added and incubated for 15 minutes. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated as described in step A. DNA was digested for 4 hours at 37°C  
 5 with 5 units of Clal in 100  $\mu$ l REact 1 buffer containing 100  $\mu$ g/ml BSA, followed by phenol extraction and precipitation of DNA as outlined in step A. The DNA precipitate was dissolved in water at a concentration of approximately 20 ng/ $\mu$ l. This Clal fragment contains the HIS4 gene and 3' AOX1 second insertable sequence.

Approximately 100 ng (10  $\mu$ l) of Clal cleaved-phosphatased pXHSA113 $\Delta$ Cla was mixed with approximately 80 ng of PstI digested-phosphatased and Clal-cleaved pA0807N (4  $\mu$ l), 4  $\mu$ l of 5X ligase buffer, 2  $\mu$ l of 1 mg/ml BSA and ligated overnight at 4°C using 1 unit of T<sub>4</sub> DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pHSA313. The pHSA313 plasmid from this ligation contains the complete pXHSA113 $\Delta$ Cla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from pA0807N. The relative orientation of the components of the pHSA313 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.  
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#### Example VII

##### Construction of 5' & 3' exact HSA expression plasmid pHSA413

20 The pHSA413 vector was constructed to provide a vector with an exact linkage between the 3' end of the AOX1 5' regulatory region and the start codon of the HSA structural gene as well as an exact linkage between the 5' end of the AOX1 3' termination sequence and the 3'end of the HSA structural gene.  
 25

##### A.. Creation of pXXHSA113 $\Delta$ Cla

1  $\mu$ g of pXHSA113 $\Delta$ Cla was digested for 4 hours at 37°C with 10 units of EcoRI in 100  $\mu$ l REact 3 buffer. The digestion mixture was phenol extracted and DNA precipitated as detailed in Example VI. DNA precipitate was dissolved in 20  $\mu$ l water and digested for 1 hour at 37°C with 20 units of Bsu36I in 100  $\mu$ l of Bsu36I buffer. The digestion mixture was phenol extracted, DNA precipitated and dissolved in 100  $\mu$ l of water as detailed in Example VI. Approximately 100 ng of EcoRI and Bsu36I-cleaved DNA was mixed with 10 ng of annealed oligonucleotides 5'-TTAGGCTTATAAG (SEQ ID NO:10) and 5'-AATTCTTATAAGCC (SEQ ID NO:11) and ligated overnight at 4°C in 20  $\mu$ l of T<sub>4</sub> DNA ligase buffer containing 100  $\mu$ g/ml BSA and 10 units of T<sub>4</sub> DNA ligase. The ligation mixture was used to transform E. coli to obtain pXXHSA113 $\Delta$ Cla. In this plasmid the sequence between Bsu36I and EcoRI (SEQ ID NO:12) present in pXHSA113 $\Delta$ Cla shown below:  
 30  
 35

Bsu36I  
 40 5' CCTTAGGCTTATAACATCTCTACATTAAAAGCATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGATCA  
 AAAGCTTATTCATCTGTGTTTCTTTCTGTTGGTGAAAGCCAACACCCGTCTAAAAAACATAAATTCTTTAATC  
 ATTTGCCTCTTTCTCTGCTTCATTAATAAAAAATGGAAAGAATCTAAAAAAAAAAAAAGGAATTC  
 45 EcoRI

is replaced by 5'CC TTA GGC TTA TAA GAATTG (SEQ ID NO:13)

Bsu36I EcoRI

50

##### B. Creation of pHSA413

55 1  $\mu$ g of pXXHSA113 $\Delta$ Cla was digested for 4 hours at 37°C with Clal in 100  $\mu$ l of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in 200  $\mu$ l reaction volume for 30 minutes at 37°C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in

water at a concentration of approximately 10 ng/ $\mu$ l as described in step A and stored at -20°C.

Approximately 100 ng (10  $\mu$ l) of ClaI cleaved-phosphatased pXXHSA113 $\Delta$ Cla was mixed with approximately 80 ng (4  $\mu$ l) of PstI digested phosphatased and ClaI-cleaved pA0807N (see paragraph 2 in step 3 of Example VI), 4  $\mu$ l of 5X ligase buffer, 2  $\mu$ l of 1 mg/ml BSA and ligated overnight at 4°C using 1 unit of T<sub>4</sub> DNA ligase. The ligation mixture was used to transform *E. coli* DG75' to obtain pHSA413. The pHSA413 plasmid from this ligation contains the complete pXXHSA113 $\Delta$ Cla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from pA0807N. The relative orientation of the components of the pHSA413 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.

10 Example VIII

Transformation of Pichia pastoris with pHSA313 and pHSA413

A. Vector preparation

15 About 10  $\mu$ g each of pHSA313, pHSA413, and pA0807N (negative control) were digested for 12 hours at 37°C in 200  $\mu$ l of HS buffer with 50 units of NotI. The digested DNA samples were phenol extracted, precipitated as described in Example VI, dissolved in 20  $\mu$ l of CaS, and were then used for transformation of *Pichia pastoris* GS115. About 10  $\mu$ g each of pHSA313, pHSA413, and pA0807N were also digested with 20 20 units of SstI for 12 hours at 37°C in 200  $\mu$ l of REact 2 buffer containing 100  $\mu$ g/ml of BSA. The digested DNA samples were extracted with phenol, precipitated as described in Example VI and dissolved in 20  $\mu$ l of CaS.

B. Cell Growth

25 *Pichia pastoris* GS115 (NRRL Y-15851) was inoculated into about 10 ml of YPD medium and shake cultured at 30°C for 12-20 hours. 100 ml of YPD medium was inoculated with a seed culture to give an OD<sub>600</sub> of about 0.001. The medium was cultured in a shake flask at 30°C for about 12-20 hours. The culture was harvested when the OD<sub>600</sub> was about 0.2-0.3 by centrifugation at 1555 g for 5 minutes using a 30 Sorvall RB5C.

C. Preparation of Spheroplasts

35 The cells were washed in 10 ml of sterile water, and then centrifuged at 1500 g for 5 minutes. (Centrifugation is performed after each cell wash at 1500 g for 5 minutes using a Sorvall RT6000B unless otherwise indicated.) The cells were washed once in 10 ml of freshly prepared SED, once in 10 ml of sterile 1M sorbitol, and finally resuspended in 10 ml of SCE buffer. 7.5  $\mu$ l of 3 mg/ml Zymolyase (100,000 units/g, obtained from Miles Laboratories) was added to the cell suspension. The cells were incubated at 30°C for about 10 minutes. (A reduction of 60% in OD<sub>600</sub> in 5% SDS can be utilized as a correct time marker.) The 40 spheroplasts were washed in 10 ml of sterile 1 M sorbitol by centrifugation at 700 g for 5-10 minutes. 10 ml of sterile CaS was used as a final cell wash, and the cells were centrifuged again at 700 g for 5-10 minutes and then resuspended in 0.6 ml of CaS.

D. Transformation

45 *Pichia pastoris* GS115 cells were transformed with 10  $\mu$ g of linearized DNA (see step A) using the spheroplast transformation technique of Sreekrishna et al, Gene 59, 115-125 (1987). DNA samples were added (up to 20  $\mu$ l volume) to 12 x 75 mm sterile polypropylene tubes. (DNA should be in a suitable buffer such as TE buffer or CaS.) 100  $\mu$ l of spheroplasts were added to each DNA sample and incubated at room 50 temperature for about 20 minutes. 1 ml of PEG solution was added to each sample and incubated at room temperature for about 15 minutes and centrifuged at 700 g for 5-10 minutes. SOS (150  $\mu$ l) was added to the pellet and incubated for 30 minutes at room temperature. Finally 850  $\mu$ l of 1M sorbitol was added.

E. Regeneration of Spheroplasts

55 A bottom agarose layer of 20 ml of regeneration agar SDR was poured per plate at least 30 minutes before transformation samples were ready. In addition, 8 ml aliquots of regeneration agar were distributed to 15 ml conical bottom Corning tubes in a 45°C water bath during the period that transformation samples

were in SOS. Aliquots of 50 or 250 µl of the transformed sample was added to the 8 ml aliquots of molten regeneration agar held at 45 °C and poured onto plates containing the solid 20 ml bottom agar layer. The plates were incubated at 30 °C for 3-5 days.

5    F. Selection of Transformants

Transformants were selected for by culturing on SDR, a media lacking histidine. The colonies which grew in the absence of histidine were also screened for "methanol-slow" phenotype, indicating displacement of the AOX1 structural gene by the NotI DNA fragment) in the case of transformants obtained using 10 NotI linearized vectors. Several transformed GS115 cells showing "methanol-normal" (those obtained with SstI linearized DNA) and methanol-slow were then cultured and assayed for the production of HSA.

Example IX

15    Methanol induced secretion of HSA in GS115/pHSA313, and GS115/pHSA413 Integrative Transformants

20    *Pichia pastoris* GS115 strains transformed with pHSA313 and pHSA413 were analysed for HSA secretion in shake tube cultures. Both methanol-slow and methanol-normal strains were used. In each case 36 independent clones were studied. Transformants obtained with pA0807N served as negative controls. A protocol was developed to ensure efficient secretion and stable accumulation of HSA in the culture medium.

25    Cells were grown to saturation in 10 ml BMGR or BMGY, and were placed in 50 ml tubes (2-3 days). The cells would be in the range of 10-20 A<sub>600</sub> units. The cells were harvested, the supernatant liquid was discarded, and then the pellet was resuspended in 2 ml of BMMR or BMMY. The tube was covered with a sterile gauze (cheese cloth) instead of a cap. The tube(s) were then returned to a 30 °C shaker. At the end 30 of 2-3 days, the cells were pelleted, and the supernatant assayed for product. The pellets could be resuspended with fresh medium and returned to the shaker for renewed secretion. With *Pichia*-HSA strains, 10 µl of media supernatant was sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under these conditions a single band of 67 kD corresponding to HSA was observed. There was no significant difference between the expression levels of GS115/pHSA313 vs GS115/pHSA413 transformants, suggesting 35 that deleting the 3' untranslated sequences from the HSA gene present in pHSA313 did not significantly affect expression levels. No significant difference in the HSA expression level was observed between methanol-slow vs methanol-normal transformants, in shake flask cultures. This suggests that disruption of AOX1 was not essential for efficient HSA expression. As expected, HSA was absent in both the culture medium and the cell extract of GS115/pA0807N transformants (negative control).

35    Example X

Batch-Fed Fermentation of Mut<sup>-</sup> *Pichia pastoris* for Production of HSA

40    *Pichia pastoris* GS115:pHSA 413-6 was inoculated into a 20 liter Biolafitte fermentor with an 8.5 l working volume. The inoculum was prepared in the following manner: a culture was grown on a YM plate and then transferred to 100 ml YM broth in a shake flask and grown for about 24 hours. 50 mls of this culture was transferred to 1 liter of YM broth in a shake flask and also grown for about 24 hours. 1 liter of this was then transferred to 8.5 liters of fermentor medium in the Biolafitte fermentor. Fermentor medium 45 consisted of Minimal salts + biotin + 5 percent glycerol. Batch growth conditions included the following: pH = 5.8 (controlled with NH<sub>3</sub>), temperature = 30 ° C, and percent dissolved oxygen greater than 20 percent air saturation.

50    Glycerol exhaustion was complete after about 24 hours, at which time a slow methanol feed was begun at a rate of 10-15 ml/hr. The methanol concentration was monitored in the fermentor and the feed rate was adjusted to maintain a concentration of 0.5-0.9 percent of methanol in the broth.

Secreted HSA in the media was measured quantitatively by desitometry of Coomassie blue stained polyacrylamide gels containing SDS (SDS-PAGE). Areas were referenced to a series of known weights of authentic HSA run on the same SDS-PAGE gels. The data from these gels is included in Table III.

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Table III

Production of HSA by Batch-Fed Fermentation					
Run	Strain	Run pH	Hrs. MeOH	Dry Cell Wt.	HSA in Broth g/l
1	GS115:pHSA 413-6	5.79	101	ND	2.13
2	GS115:pHSA 413-6	5.85	237	101	3.39
3	GS115:pHSA 413-6	5.85	265	98.12	2.70
4	GS115:pHSA 413-6	5.97	258	117	2.90

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: Kotikanyadan Sreekrishna et al.

10 (ii) TITLE OF INVENTION: Expression of Human Serum Albumin in  
Pichia pastoris

15 (iii) NUMBER OF SEQUENCES: 13

17 (iv) CORRESPONDENCE ADDRESS:

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20 (C) CITY: Bartlesville  
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(E) COUNTRY: USA  
25 (F) ZIP: 74005

30 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM PC  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
35 (D) SOFTWARE: Display Write 4

40 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

45 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hal Brent Woodrow  
(B) REGISTRATION NUMBER: 32,501  
(C) REFERENCE/DOCKET NUMBER: 32747

50 (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 1-918-661-0624

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 940 bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	AGATCTAAC	TCCAAAGACG	AAAGGTTGAA	TGAAACCTT	TTGCCATCCG	ACATCCACAG	60
20	GTCCATTCTC	ACACATAAGT	GCCAAACGCA	ACAGGAGGGG	ATACACTAGC	AGCAGACCGT	120
	TGCAAACGCA	GGACCTCCAC	TCCTCTTCTC	CTCAACACCC	ACTTTGCCA	TCGAAAACC	180
	AGCCCAGTTA	TTGGGCTTGA	TTGGAGCTCG	CTCATTCCA	TTCCCTCTAT	TAGGCTACTA	240
25	ACACCATGAC	TTTATTAGCC	TGTCTATCCT	GGCCCCCCTG	GCGAGGTTC	TGTTTGT	300
	TTTCCGAATG	CAACAAGCTC	CGCATTACAC	CCGAACATCA	CTCCAGATGA	GGGCTTCTG	360
	AGTGTGGGT	CAAATAGTTT	CATGTTCCCC	AAATGGCCCA	AAACTGACAG	TTTAAACGCT	420
30	GTCTTGGAAC	CTAATATGAC	AAAAGCGTGA	TCTCATCAA	GATGAACTAA	GTTTGGTTCG	480
	TTGAAATGCT	AACGGCCAGT	TGGTCAAAAA	GAAACTTCCA	AAAGTCGGCA	TACCGTTGT	540
	CTTGTGGGT	ATTGATTGAC	GAATGCTCAA	AAATAATCTC	ATTAATGCTT	AGCGCAGTCT	600
35	CTCTATCGCT	TCTGAACCCC	GGTGCACCTG	TGCCGAAACG	CAAATGGGGA	AACACCCGCT	660
	TTTGGATGA	TTATGCATTG	TCTCCACATT	GTATGCTTCC	AAGATTCTGG	TGGGAATACT	720
40	GCTGATAGCC	TAACGTTCAT	GATCAAAATT	TAACTGTTCT	AACCCCTACT	TGACAGCAAT	780
	ATATAAACAG	AAGGAAGCTG	CCCTGTCTTA	AACCTTTTT	TTTATCATCA	TTATTAGCTT	840
	ACTTTCTAA	TTGCGACTGG	TTCCAATTGA	CAAGCTTTG	ATTTAACGA	CTTTAACGA	900
45	CAACTTGAGA	AGATCAAAAA	ACAACTAATT	ATTCGAAACG			940

## (3) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

5                   (A) LENGTH: 600 bp  
                  (B) TYPE: nucleic acid  
                  (C) STRANDEDNESS: single  
                  (D) TOPOLOGY: linear

## 10                  (ii) MOLECULE TYPE: Genomic DNA

## 15                  (ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAAGTAAACC	CCATTCAATG	TTCCGAGATT	TAGTATACTT	GCCCCTATAA	GAAACGAAGG	60
ATTCAGCTT	CCTTACCCCA	TGAACAGAAA	TCTTCCATT	ACCCCCACT	GGAGAGATCC	120
GCCCAAACGA	ACAGATAATA	GAAAAAAAGAA	ATTCGGACAA	ATAGAACACT	TTCTCAGCCA	180
ATTAAGTCA	TTCCATGCAC	TCCCTTAGC	TGCCGTTCCA	TCCCTTGTT	GAGCAACACC	240
ATCGTTAGCC	AGTACGAAAG	AGGAAACTTA	ACCGATAACCT	TGGAGAAATC	TAAGGCGCGA	300
ATGAGTTAG	CCTAGATATC	CTTAGTGAAG	GGTGTCCGA	TACCTTCTCC	ACATTCAAGTC	360
ATAGATGGGC	AGCTTGTAA	TCATGAAGAG	ACGGAAACGG	GCATTAAGGG	TTAACCGCCA	420
AATTATATAA	AAGACAACAT	GTCCCCAGTT	TAAAGTTTT	CTTCCTATT	CTTGTATCCT	480
GAGTGACCGT	TGTGTTAAAT	ATAACAAGTT	CGTTTAACT	TAAGACCAAA	ACCAGTTACA	540
ACAAATTATA	ACCCCTCTAA	ACACTAAAGT	TCACTCTTAT	CAAACATATCA	AACATCAAAA	600

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## (4) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 1830 bp  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Genomic DNA

## 10 (ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	ATG	AAG	TGG	GTA	ACC	TTT	ATT	TCC	CTT	CTT	TTT	CTC	TTT	AGC	TCG	45
5	Met	Lys	Trp	Val	Thr	Phe	Ile	Ser	Leu	Leu	Phe	Leu	Phe	Ser	Ser	
	-35						-30					-25				
15	GCT	TAT	TCC	AGG	GGT	GTG	TTT	CGT	CGA	GAT	GCA	CAC	AAG	AGT	GAG	90
	Ala	Tyr	Ser	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His	Lys	Ser	Glu	
	-20						-15					-10				
20	GTT	GCT	CAT	CGG	TTT	AAA	GAT	TTG	GGA	GAA	GAA	AAT	TTC	AAA	GCC	135
	Val	Ala	His	Arg	Phe	Lys	Asp	Leu	Gly	Glu	Glu	Asn	Phe	Lys	Ala	
	-5						1					5				
25	TTG	GTG	TTG	ATT	GCC	TTT	GCT	CAG	TAT	CTT	CAG	CAG	TGT	CCA	TTT	180
	Leu	Val	Leu	Ile	Ala	Phe	Ala	Gln	Tyr	Leu	Gln	Gln	Cys	Pro	Phe	
	10						15					20				
30	GAA	GAT	CAT	GTA	AAA	TTA	GTG	AAT	GAA	GTA	ACT	GAA	TTT	GCA	AAA	225
	Glu	Asp	His	Val	Lys	Leu	Val	Asn	Glu	Val	Thr	Glu	Phe	Ala	Lys	
	25						30					35				
35	ACA	TGT	GTT	GCT	GAT	GAG	TCA	GCT	GAA	AAT	TGT	GAC	AAA	TCA	CTT	270
	Thr	Cys	Val	Ala	Asp	Glu	Ser	Ala	Glu	Asn	Cys	Asp	Lys	Ser	Lue	
	40						45					50				
40	CAT	ACC	CTT	TTT	GGA	GAC	AAA	TTA	TGC	ACA	GTT	GCA	ACT	CTT	CGT	315
	His	Thr	Leu	Phe	Gly	Asp	Lys	Leu	Cys	Thr	Val	Ala	Thr	Leu	Arg	
	55						60					65				
45	GAA	ACC	TAT	GGT	GAA	ATG	GCT	GAC	TGC	TGT	GCA	AAA	CAA	GAA	CCT	360
	Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	Lys	Gln	Glu	Pro	
	70						75					80				
50	GAG	AGA	AAT	GAA	TGC	TTC	TTG	CAA	CAC	AAA	GAT	GAC	AAC	CCA	AAC	405
	Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	Asn	Pro	Asn	
	85						90					95				
45	CTC	CCC	CGA	TTG	GTG	AGA	CCA	GAG	GTT	GAT	GTG	ATG	TGC	ACT	GCT	450
	Leu	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	Thr	Ala	
	100						105					110				
50	TTT	CAT	GAC	AAT	GAA	GAG	ACA	TTT	TTG	AAA	AAA	TAC	TTA	TAT	GAA	495
	Phe	His	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	Glu	
	115						120					125				

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	ATT	GCC	AGA	AGA	CAT	CCT	TAC	TTT	TAT	GCC	CCG	GAA	CTC	CTT	TTC	540
	Ile	Ala	Arg	Arg	His	Pro	Tyr	Phe	Tyr	Ala	Pro	Glu	Leu	Leu	Phe	
	130					135						140				
5	TTT	GCT	AAA	AGG	TAT	AAA	GCT	GCT	TTT	ACA	GAA	TGT	TGC	CAA	GCT	585
	Phe	Ala	Lys	Arg	Tyr	Lys	Ala	Ala	Phe	Thr	Glu	Cys	Cys	Gln	Ala	
	145					150					155					
10	GCT	GAT	AAA	GCT	GCC	TGC	CTG	TTG	CCA	AAG	CTC	GAT	GAA	CTT	CGG	630
	Ala	Asp	Lys	Ala	Ala	Cys	Leu	Leu	Pro	Lys	Leu	Asp	Glu	Leu	Arg	
	160					165					170					
15	GAT	GAA	GGG	AAG	GTT	TCG	TCT	GCC	AAA	CAG	AGA	CTC	AAG	TGT	GCC	675
	Asp	Glu	Gly	Lys	Val	Ser	Ser	Ala	Lys	Gln	Arg	Leu	Lys	Cys	Ala	
	175					180				185						
20	AGT	CTC	CAA	AAA	TTT	GGA	GAA	AGA	GCT	TTC	AAA	GCA	TGG	GCA	GTA	720
	Ser	Leu	Gln	Lys	Phe	Gly	Glu	Arg	Ala	Phe	Lys	Ala	Trp	Ala	Val	
	190					195				200						
25	GCT	CGC	CTG	AGC	CAG	AGA	TTT	CCC	AAA	GCT	GAG	TTT	GCA	GAA	GTT	765
	Ala	Arg	Leu	Ser	Gln	Arg	Phe	Pro	Lys	Ala	Glu	Phe	Ala	Glu	Val	
	205					210				215						
30	TCC	AAG	TTA	GTG	ACA	GAT	CTT	ACC	AAA	GTC	CAC	ACG	GAA	TGC	TGC	810
	Ser	Lys	Leu	Val	Thr	Asp	Leu	Thr	Lys	Val	His	Thr	Glu	Cys	Cys	
	220					225				230						
35	CAT	GGA	GAT	CTG	CTT	GAA	TGT	GCT	GAT	GAC	AGG	GCG	GAC	CTT	GCC	855
	His	Gly	Asp	Leu	Leu	Glu	Cys	Ala	Asp	Asp	Arg	Ala	Asp	Leu	Ala	
	235					240				245						
40	AAG	TAT	ATC	TGT	GAA	AAT	CAA	GAT	TCG	ATC	TCC	AGT	AAA	CTG	AAG	900
	Lys	Tyr	Ile	Cys	Glu	Asn	Gln	Asp	Ser	Ile	Ser	Ser	Lys	Leu	Lys	
	250					255				260						
45	GAA	TGC	TGT	GAA	AAA	CCT	CTG	TTG	GAA	AAA	TCC	CAC	TGC	ATT	GCC	945
	Glu	Cys	Cys	Glu	Lys	Pro	Leu	Leu	Glu	Lys	Ser	His	Cys	Ile	Ala	
	265					270				275						
50	GAA	GTG	GAA	AAT	GAT	GAG	ATG	CCT	GCT	GAC	TTG	CCT	TCA	TTA	GCT	990
	Glu	Val	Glu	Asn	Asp	Glu	Met	Pro	Ala	Asp	Leu	Pro	Ser	Leu	Ala	
	280					285				290						
55	GCT	GAT	TTT	GTT	GAA	AGT	AAG	GAT	GTT	TGC	AAA	AAC	TAT	GCT	GAG	1035
	Ala	Asp	Phe	Val	Glu	Ser	Lys	Asp	Val	Cys	Lys	Asn	Tyr	Ala	Glu	
	295					300				305						
55	GCA	AAG	GAT	GTC	TTC	TTG	GGC	ATG	TTT	TTG	TAT	GAA	TAT	GCA	AGA	1080
	Ala	Lys	Asp	Val	Phe	Leu	Gly	Met	Phe	Leu	Tyr	Glu	Tyr	Ala	Arg	
	310					315				320						

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	AGG	CAT	CCT	GAT	TAC	TCT	GTC	GTG	CTG	CTG	CTG	AGA	CTT	GCC	AAG	1125
	Arg	His	Pro	Asp	Tyr	Ser	Val	Val	Leu	Leu	Leu	Leu	Arg	Leu	Ala	Lys
	325					330						335				
5	ACA	TAT	GAA	ACC	ACT	CTA	GAG	AAG	TGC	TGT	GCC	GCT	GCA	GAT	CCT	1170
	Thr	Tyr	Glu	Thr	Thr	Leu	Glu	Lys	Cys	Cys	Ala	Ala	Ala	Ala	Asp	Pro
	340				345						350					
10	CAT	GAA	TGC	TAT	GCC	AAA	GTG	TTC	GAT	GAA	TTT	AAA	CCT	CTT	GTG	1215
	His	Glu	Cys	Tyr	Ala	Lys	Val	Phe	Asp	Glu	Phe	Lys	Pro	Leu	Val	
	355				360						365					
15	GAA	GAG	CCT	CAG	AAT	TTA	ATC	AAA	CAA	AAT	TGT	GAG	CTT	TTT	GAG	1260
	Glu	Glu	Pro	Gln	Asn	Leu	Ile	Lys	Gln	Asn	Cys	Glu	Leu	Phe	Glu	
	370				375						380					
20	CAG	CTT	GGA	GAG	TAC	AAA	TTC	CAG	AAT	GCG	CTA	TTA	GTT	CGT	TAC	1305
	Gln	Leu	Gly	Glu	Tyr	Lys	Phe	Gln	Asn	Ala	Leu	Leu	Val	Arg	Tyr	
	385				390						395					
25	TCA	AGA	AAC	CTA	GGA	AAA	GTG	GGC	AGC	AAA	TGT	TGT	AAA	CAT	CCT	1395
	Ser	Arg	Asn	Leu	Gly	Lys	Val	Gly	Ser	Lys	Cys	Cys	Lys	His	Pro	
	415				420						425					
30	GAA	GCA	AAA	AGA	ATG	CCC	TGT	GCA	GAA	GAC	TAT	CTA	TCC	GTG	GTC	1440
	Glu	Ala	Lys	Arg	Met	Pro	Cys	Ala	Glu	Asp	Tyr	Leu	Ser	Val	Val	
	430				435						440					
35	CTG	AAC	CAG	TTA	TGT	GTG	TTG	CAT	GAG	AAA	ACG	CCA	GTA	AGT	GAC	1485
	Leu	Asn	Gln	Leu	Cys	Val	Leu	His	Glu	Lys	Thr	Pro	Val	Ser	Asp	
	445				450						455					
40	AGA	GTC	ACC	AAA	TGC	TGC	ACA	GAA	TCC	TTG	GTG	AAC	AGG	CGA	CCA	1530
	Arg	Val	Thr	Lys	Cys	Cys	Thr	Glu	Ser	Leu	Val	Asn	Arg	Arg	Pro	
	460				465						470					
45	TGC	TTT	TCA	GCT	CTG	GAA	GTC	GAT	GAA	ACA	TAC	GTT	CCC	AAA	GAG	1575
	Cys	Phe	Ser	Ala	Leu	Glu	Val	Asp	Glu	Thr	Tyr	Val	Pro	Lys	Glu	
	475				480						485					
50	TTT	AAT	GCT	GAA	ACA	TTC	ACC	TTC	CAT	GCA	GAT	ATA	TGC	ACA	CTT	1620
	Phe	Asn	Ala	Glu	Thr	Phe	Thr	Phe	His	Ala	Asp	Ile	Cys	Thr	Leu	
	490				495						500					
	TCT	GAG	AAG	GAG	AGA	CAA	ATC	AAG	AAA	CAA	ACT	GCA	CTT	GTT	GAG	1665
	Ser	Glu	Lys	Glu	Arg	Gln	Ile	Lys	Lys	Gln	Thr	Ala	Leu	Val	Glu	
	505				510						515					

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	CTT	G TG	AAA	CAC	AAG	CCC	AAG GCA	ACA AAA	GAG CAA	CTG AAA	GCT	1710
	Leu	Val	Lys	His	Lys	Pro	Lys Ala	Thr Lys	Glu Gln	Leu Lys	Ala	
520						525				530		
5	GTT	ATG	GAT	GAT	TTC	GCA	GCT TTT	GTA GAG	AAG TGC	TGC AAG	GCT	1755
	Val	Met	Asp	Asp	Phe	Ala	Ala Phe	Val Glu	Lys Cys	Cys Lys	Ala	
535						540				545		
10	GAC	GAT	AAG	GAG	ACC	TGC	TTT GCC	GAG GAG	GGT AAA	AAA CTT	GTT	1800
	Asp	Asp	Lys	Glu	Thr	Cys	Phe Ala	Glu Gly	Lys Lys	Lys Leu	Val	
	550					555				560		
15	GCT	GCA	AGT	CAA	GCT	GCC	TTA GGC	TTA TAA				1830
	Ala	Ala	Ser	Gln	Ala	Ala	Leu Gly	Leu				
	565					570						

(5) INFORMATION FOR SEQ ID NO:4:

- 20           (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36bp
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- 25           (ii) MOLECULE TYPE: Oligonucleotide
- (ix) SEQUENCE DESCRIPTION: SEQ ID NO:4:

30	CCCTCACACG	CCTTGATT	C	ATG	AAG	TGG	GTA	ACC	36
	Met	Lys	Trp	Val	Thr				
	1					5			

(6) INFORMATION FOR SEQ ID NO:5:

- 35           (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39bp
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- 40           (ii) MOLECULE TYPE: Oligonucleotide
- (ix) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45	GCC	TTA	GGC	TTA	TAAGAATTCA	GTAAAGC	ATCTCAG	39
	Ala	Leu	Gly	Leu				
	570							

50

55

(7) INFORMATION FOR SEQ ID NO:6:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: Screening Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:6:

15 GCCTGGGAAT CC ATG AAG 18

Met Lys

1

(8) INFORMATION FOR SEQ ID NO:7:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: Screening Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30 TTA TAAGAATTCA GTTTA 18

Leu

573

35 (9) INFORMATION FOR SEQ ID NO:8:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16bp
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:8:

50 CGAAACG ATG AAG TGG 16

Met Lys Trp

(10) INFORMATION FOR SEQ ID NO:9:

5  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19bp  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10  
(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTTACCCACT TCATCGTTT 19

15  
(11) INFORMATION FOR SEQ ID NO:10:

20  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13bp  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25  
(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTAGGCTTAT AAG 13

30  
(12) INFORMATION FOR SEQ ID NO:11:

35  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14bp  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

40  
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATTCTTATA AGCC 14

45

50

55

## (13) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

5                   (A) LENGTH: 231bp  
                  (B) TYPE: nucleic acid  
                  (C) STRANDEDNESS: single  
                  (D) TOPOLOGY: linear

10                   (ii) MOLECULE TYPE: Linker Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTTAGGCTT	ATAACATCTC	TACATTAAA	AGCATTCAG	CCTACCAGA	GAATAAGAGA	60
AAGAAAATGA	AGATCAAAAG	CTTATTCTAC	TGTGTTTCT	TTTCGGTTGG	TGTAAAGCCA	120
ACACCCCTGTC	TAAAAAACAT	AAATTTCTTT	AATCATTTTG	CCTCTTTTC	TCTGTGCTTC	180
AATTAATAAA	AAATGGAAAG	AATCTAAAAA	AAAAAAAAAA	AAAAGGAATT	C	231

## (14) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

25                   (A) LENGTH: 20bp  
                  (B) TYPE: nucleic acid  
                  (C) STRANDEDNESS: single  
                  (D) TOPOLOGY: linear

30                   (ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35                   CCTTAGGCTT ATAAGAATT 20

## Claims

40       1. An expression cassette for the production of HSA in *Pichia pastoris* comprising  
          a) a *Pichia pastoris* 5' regulatory region having a 5' end and a 3' end selected from a *Pichia pastoris* AOX1 5' regulatory region and a *Pichia pastoris* DAS1 5' regulatory region wherein the 3' end of the 5' regulatory region is operably linked to  
          b) a HSA structural gene encoding a HSA signal sequence and a mature HSA protein, having a 5' end and a 3' end wherein the HSA structural gene has an ATG start codon within no more than 11 deoxyribonucleotides of the 5' end of said HSA structural gene; operably linked to  
          c) a suitable 3' termination sequence.

45       2. The expression cassette of claim 1 wherein the 5' regulatory region is selected from the AOX1 5' regulatory region and the DAS1 5' regulatory region from *Pichia pastoris*.

50       3. The expression cassette of claim 1 wherein the 3' termination sequence is isolated from a *Pichia pastoris* gene selected from an AOX1 gene, a DAS1 gene, a p40 gene and a HIS4 gene.

55       4. The expression cassette of claim 1 wherein the expression cassette is incorporated into a vector selected from circular plasmids and linear plasmids, the latter preferably being integrative site-specific vectors.

5. The expression cassette of claim 1 wherein the expression cassette is incorporated into a vector comprising the following serial arrangement:

- a first insertable DNA fragment;
- at least one marker gene; and,
- c) a second insertable DNA fragment; wherein at least one expression cassette is incorporated either before or after the marker gene of component (b), and the first and second insertable DNA fragments employed are homologous with separate portions of the *Pichia pastoris* genome and the insertable fragments are in the same relative orientation as exist in the *Pichia pastoris* genome.

10 6. The expression cassette of claim 5 contained in said vector wherein the first insertable DNA fragment and the second insertable DNA fragment are obtained from the DNA sequences of a gene isolated from *Pichia pastoris* selected from an AOX1 gene, a p40 gene, a DAS1 gene and an HIS4 gene.

15 7. The expression cassette of claim 5 contained in said vector wherein the marker gene is selected from a *Pichia pastoris* HIS4 gene, a *Pichia pastoris* ARG4 gene, a *Saccharomyces cerevisiae* SUC2 gene, a G418<sup>R</sup> gene of bacterial transposon Tn601 and a G418<sup>R</sup> gene of bacterial transposon Tn903.

20 8. The expression cassette of claim 5 wherein said vector comprises

- a first insertable DNA fragment which is an operable 5' regulatory region from the AOX1 gene being about one kilobase in length isolated from *Pichia pastoris* operably linked to
- a HSA structural gene encoding a HSA signal sequence and a mature HSA protein having a 5' end and a 3' end wherein the HSA structural gene has a ATG start codon within no more than 8 deoxyribonucleotides of the 5' end of said HSA structural gene; operably linked to
- the 3' termination sequence of the AOX1 gene isolated from *Pichia pastoris*; operably linked to
- d) a marker gene which is the HIS4 gene isolated from *Pichia pastoris*; operably linked to
- e) a second insertable DNA fragment which is about 0.65 kilobases of the AOX1 3' termination sequence.

25 9. The expression cassette of claim 8 wherein the HSA structural gene has a ATG start codon with either the deoxyribonucleotide AGGAATTC or no deoxyribonucleotide 5' of said ATG start codon.

30 10. A *Pichia pastoris* cell transformed with an expression cassette according to any of claims 1 to 9.

35 11. The *Pichia pastoris* cell of claim 10 wherein the *Pichia pastoris* cell to be transformed is selected from *Pichia pastoris* GS115 (NRRL Y-15851), *Pichia pastoris* GS190 (NRRL Y-18014), *Pichia pastoris* PPF1 (NRRL Y-18017), *Pichia pastoris* (NRRL Y-11430) and *Pichia pastoris* (NRRL Y-11431), wherein *Pichia pastoris* GS 115 (NRRL Y-15851) is the most preferred *Pichia pastoris* cell.

40 12. A process for the secretion of HSA from transformed *Pichia pastoris* cells according to claim 10 or 11 comprising

- transforming a *Pichia pastoris* cell with at least one vector having at least one expression cassette according to any of claims 1 to 9, and
- culturing the resulting transformed *Pichia pastoris* cell under suitable conditions to obtain the production of HSA.

45

50

55

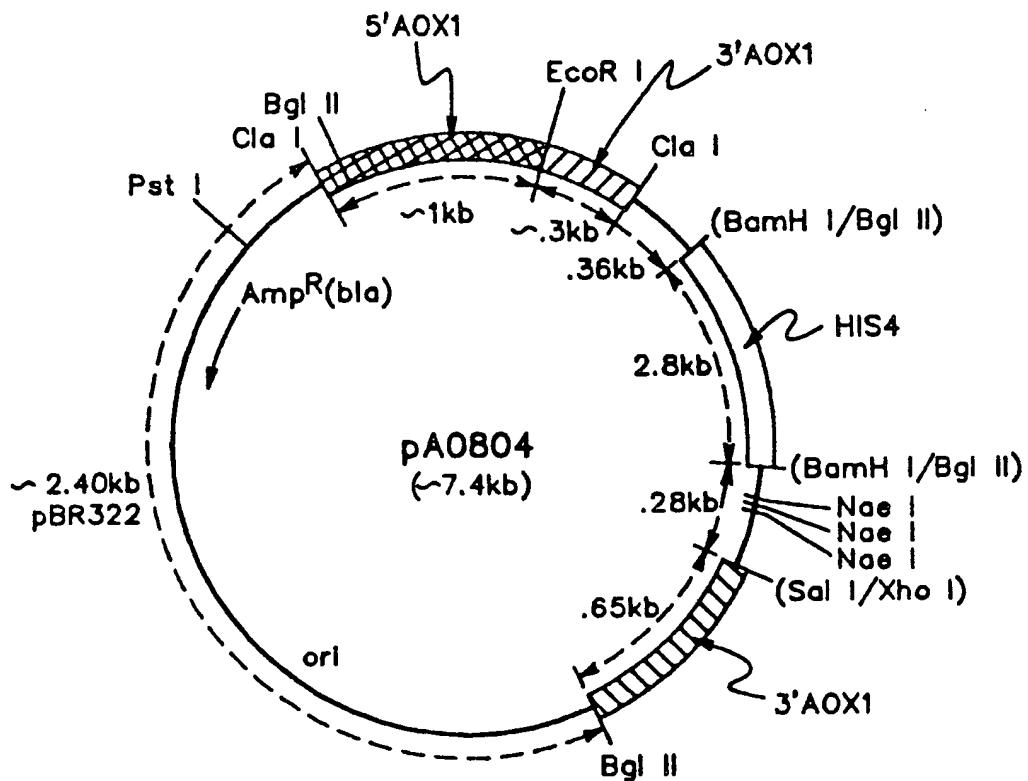
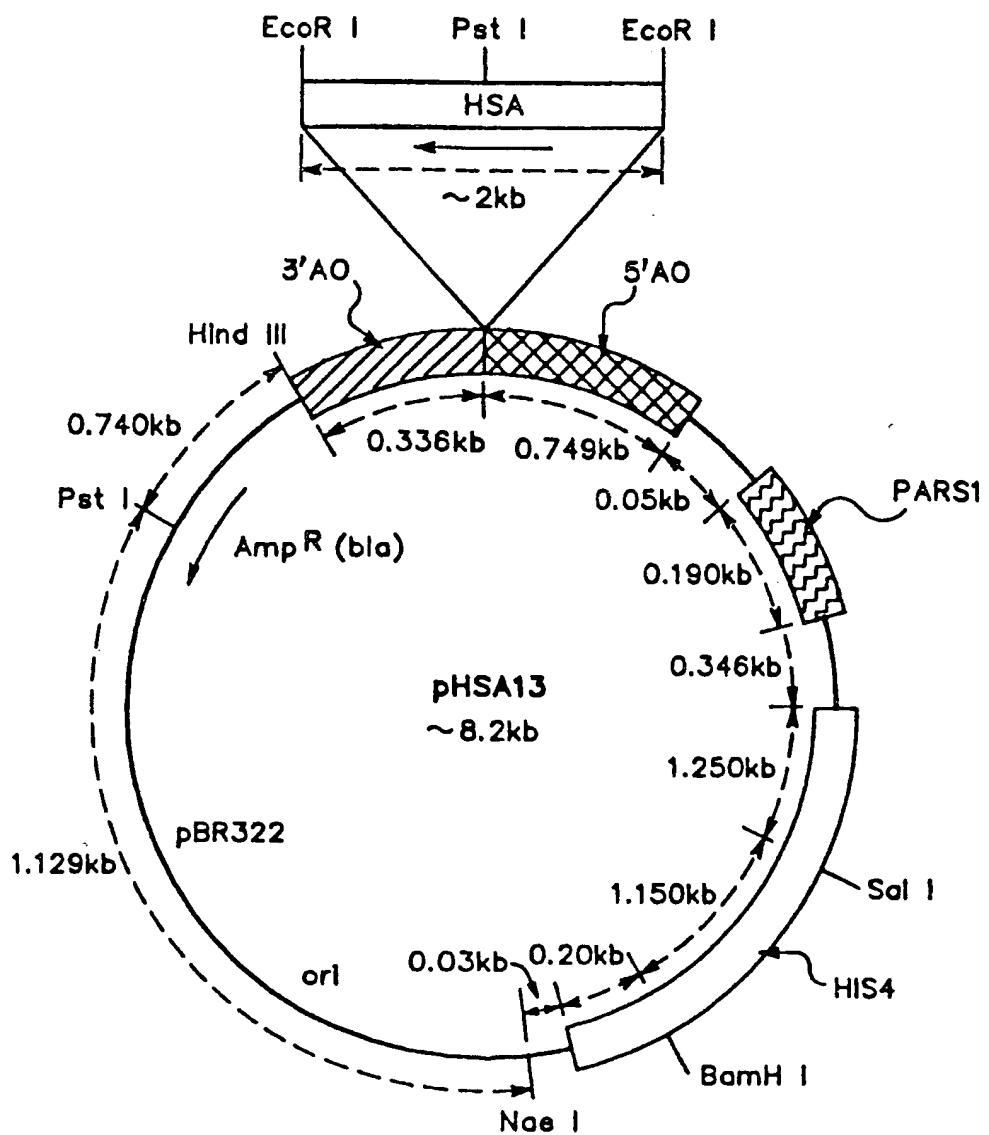
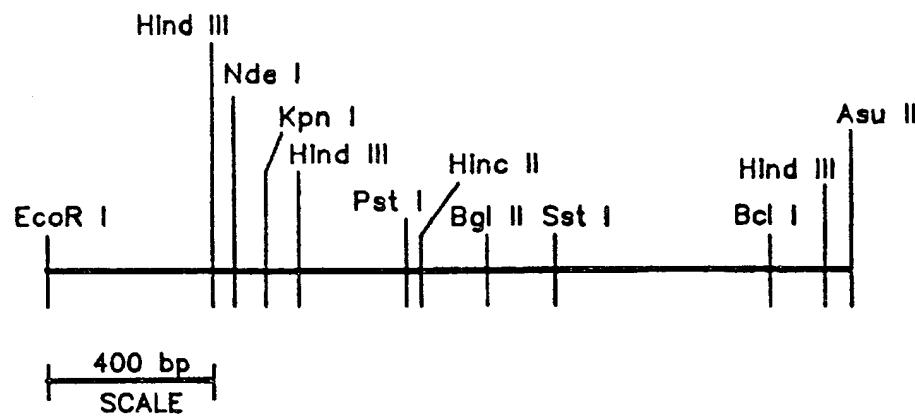
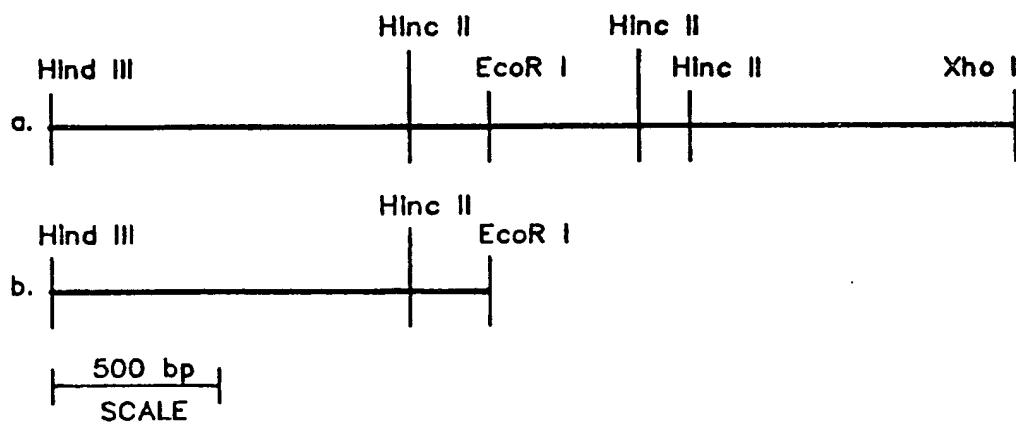


FIG. 1

**FIG. 2**



**FIG. 3**



**FIG. 4**

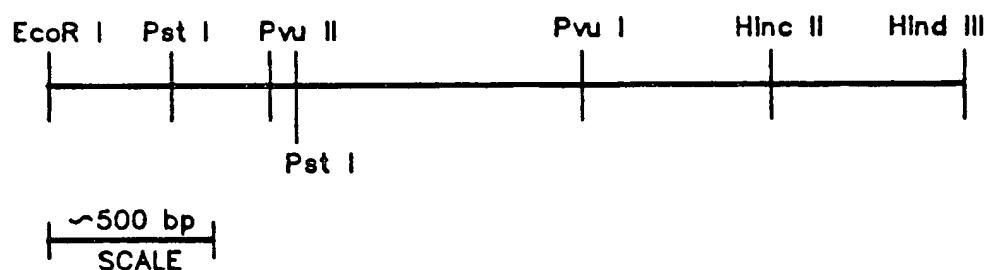


FIG. 5

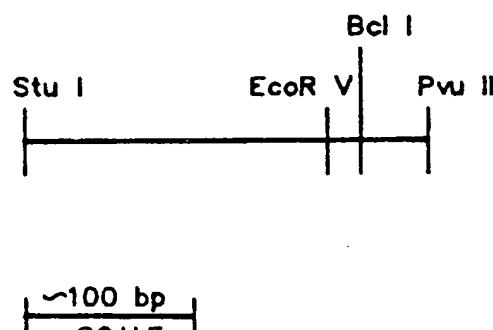


FIG. 6

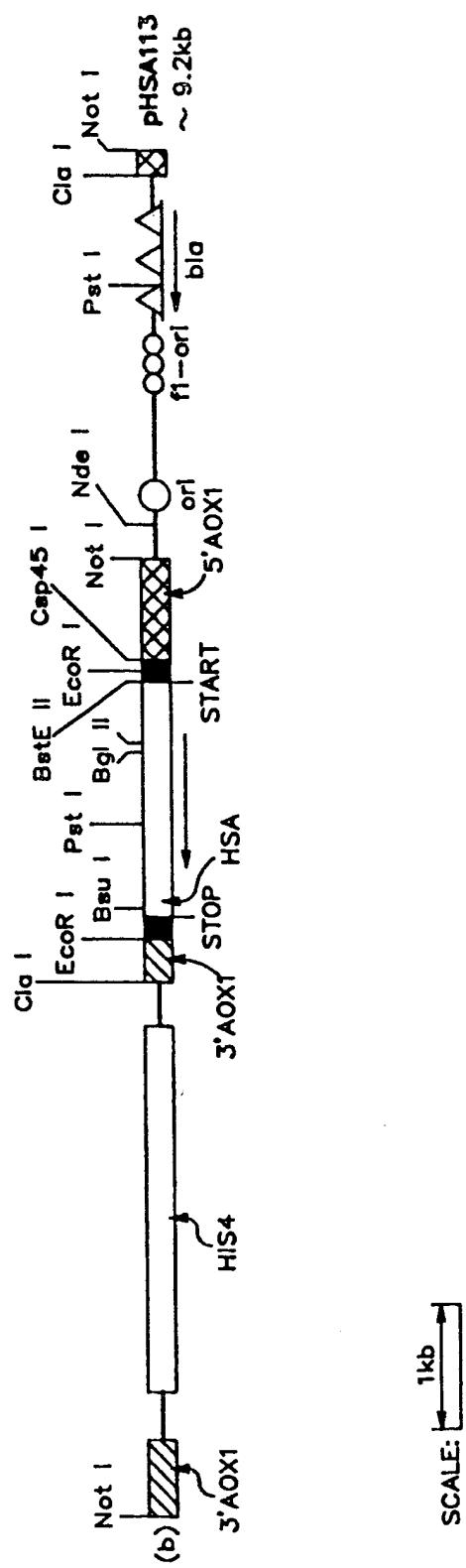


FIG. 7

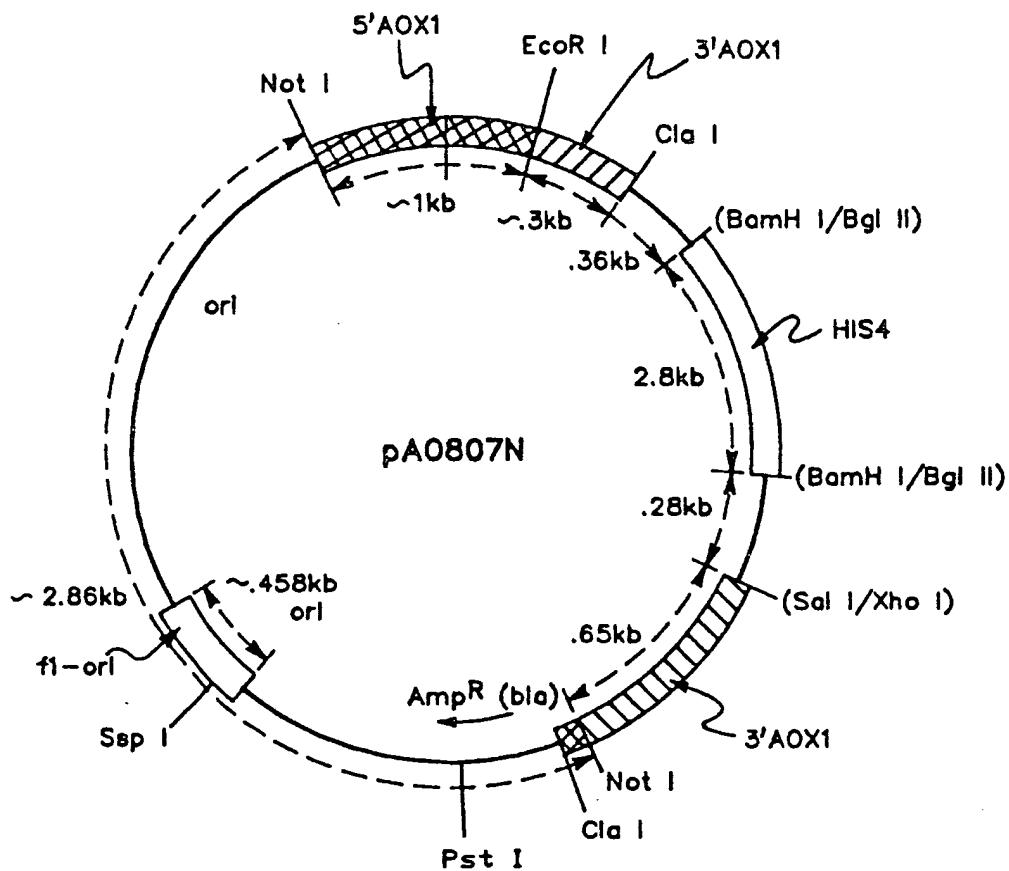


FIG. 8

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